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Stromelysin-3 (ST-3), a member of the matrix metalloproteinase (MMP) family, is expressed in stromal cells surrounding invasive breast carcinoma cells where it undergoes processing to its active form by an intracellular, proprotein convertase-dependent pathway. While strong correlations between disease progression and ST-3 expression suggest that the proteinase plays a critical role in breast cancer, human ST-3 is the only matrix metalloproteinase that does not express a marked ability to degrade components of the extracellular matrix (ECM). To further characterize the role of ST-3 *in vivo*, our attention focused on the analysis of lines of transgenic mice wherein the whey acidic protein (WAP) gene was used to direct human ST-3 expression to mammary tissue during late pregnancy through lactation. In these studies, high levels of the ST-3 transgene were generated *in vivo* which unexpectedly triggered a massive involution program. Surprisingly, ST-3 not only induced an apoptotic program, but also initiated changes in the underlying basement membrane *in vivo*. In addition, ST-3 expression also appeared to induce an exaggerated angiogenic response in involuting tissues. These data provide the first evidence that ST-3 can regulate mammary epithelial-ECM interactions and angiogenesis *in vivo*.

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I. INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by either expressing, or inducing the expression of, proteolytic enzymes that degrade structural barriers established by the extracellular matrix (ECM)[1-3]. Although the identity of the specific proteinases that lend cancer cells their invasive potential remains the subject of conjecture, attention has recently focused on the matrix-degrading metalloproteinases (MMPs) - a family of at least 15 zinc-dependent proteolytic enzymes whose overlapping substrate specificities include all of the major components of the ECM[1-5]. Consistent with their presumed role in tumor progression, *in situ* analyses of a variety of cancerous tissues have confirmed heightened levels of expression of one or more MMPs in tumor and/or surrounding stromal tissues[6-8]. Furthermore, a range of *in vitro* as well as *in vivo* studies have demonstrated that invasion and metastasis can be affected by altering MMP activity[9-12]. Given these findings, efforts have intensified to identify those MMPs that might be used as diagnostic indicators or potential targets for pharmacologic intervention in breast as well as other cancers.

Until recently, attempts to implicate specific MMPs in breast cancer progression were based on the assumption that all of the major proteinases belonging to this gene family had been identified and characterized[2]. Unexpectedly however, differential screens of breast cancer cDNA libraries led to the tentative identification of a new member of the MMP family, termed stromelysin-3, on the basis of its apparent homology to stromelysin-1 and -2 (two other members of this gene family that had been previously characterized)[13]. The expressed gene product was predicted to encode a 488-residue protein containing i) a candidate leader sequence, ii) a highly conserved PRCGXPD motif believed to maintain the latency of MMP zymogens, iii) a zinc-binding catalytic motif and iv) a carboxyl-terminal domain with sequence homology to the heme-binding protein, hemopexin and the ECM molecule, vitronectin[13]. Interestingly, in early studies all invasive breast carcinomas examined thus far, ST-3 was not a product of the neoplastic cells themselves, but rather the surrounding stromal cells[6,12,13]. However, more recent analyses have demonstrated that tumor cells themselves can express ST-3 as well[14].

Given the structural similarity between ST-3 and other members of the MMP family, it was postulated that the enzyme would be secreted as a zymogen whose extracellular activation at the tumor-stroma interface would follow the destabilization of the ligand formed between the Cys in the PRCGVPD domain and the Zn^{+2} in the catalytic domain[6,12,13]. In a scheme analogous to that established for the other MMPs, ST-3 could then under autoproteolytic processing to a mature, active form which presumably would catalyze the degradation of critical ECM components localized in either the basement membrane or stroma[4,5]. However, despite the structural similarities that exist between ST-3 and other MMP members, additional studies indicate that i) the primary sequence of ST-3 is distinct from all other members of the MMP family and ii) the assumed role of ST-3 in ECM remodeling cannot be readily confirmed[4,15]. First, comparisons of the catalytic domains of the MMPs suggest that ST-3 belongs in a new subgroup relative to all other members of this gene family and that its closest homology lies with the bacterial metalloproteinases[4]. Consistent with the contention that ST-3 is a structurally distinct entity in the MMP family, the human ST-3 gene has been localized to the long arm of chromosome 22, a position which differs from those of all other MMP genes[4,12]. Second, although the expression of the human ST-3 protein had not yet been reported, the homologous mouse enzyme (~80%

homology identity at the amino acid level) could only be isolated in a truncated form that expressed weak proteolytic activity[15]. Indeed, these results led Murphy and colleagues to conclude that "the enzyme may not function as a matrix-degrading metalloproteinase[15]. Taken together, these findings indicated that despite the provocative correlative link established between ST-3 expression and breast cancer progression, the role of this proteinase in invasive events remained undefined. In this proposal, we sought to use a series of *in vitro* as well as *in vivo* biochemical and molecular approaches to i)purify and characterize ST-3 expressed by stably-transfected mammalian cells, ii) determine the mechanism by which the ST-3 zymogen is activated and regulated, iii) determine the ability of stromelysin-3 to regulate the invasive potential of breast carcinoma cells *in vitro* and iv) characterize the role of ST-3 *in vivo* in a transgenic mouse model. To date, the first three aims have been completed and our final efforts have focused on characterizing the role of ST-3 *in vivo* in a transgenic mouse model generated in our laboratories.

II. BODY

During the previous granting periods, we completed our analysis of the ST-3/proprotein convertase axis, demonstrated that the processing motif encrypted in ST-3 may also operate in the related membrane-type matrix metalloproteinases (i.e., MT1, 2, 3, 4-MMP), and characterized the inability of ST-3-transfected cells to express an invasive phenotype *in vitro* [16-18]. While the first two findings clearly indicated that the proprotein convertases play a critical role in regulating the activities of multiple matrix metalloproteinases, the inability of active ST-3 to degrade extracellular matrix components or promote invasive behavior precluded any predictions of the enzyme's relevance to tumor progression *in vivo*. (Earlier reports by Murphy and colleagues suggesting that truncated products of ST-3 express matrix-degrading activity could not be confirmed with human ST-3. Subsequent studies demonstrated that a single amino acid substitution between human and mouse ST-3 was responsible for the differences in activity[19].) However, in our last progress report, we provided a preliminary description of an ST-3-triggered premature involution program involving mammary epithelial cell apoptosis and basement membrane remodeling and ii) an unanticipated association between ST-3 expression and angiogenesis. These studies have now been completed and the conclusions described below.

ST-3-INDUCED MAMMARY GLAND INVOLUTION

Preliminary attempts to target transgene expression specifically to the stroma using an RSV promoter were unsuccessful (see prior Progress Report). Consequently, plans to assess the effect of stroma-derived ST-3 on tumor cell growth and invasion were tabled. As an alternative, we opted to place ST-3 expression under the control of regulatory elements of the mammary epithelial cell-specific, pregnancy -responsive whey acidic protein (WAP) gene[20,21]. Because ST-3 is normally expressed during mammary gland involution (a state that closely parallels many of the matrix-remodeling events observed in carcinomatous states[22,23]) and can be expressed by carcinomas themselves[14], our rationale was to express the transgene during mid-pregnancy and through lactation in order to observe the effect exerted on the well-characterized mammary gland phenotype. (It should be noted that this approach has been used by other groups to characterize the role of stromelysin-1 in mammary gland pathophysiology[20,24]. However, despite

the similarity of their names, stromelysin-1 and -3 are not closely related gene products and display completely different mechanisms of regulation and activity[16,17]). Thus, transgenic mice were generated using a chimeric recombinant DNA composed of the WAP promoter and the entire ST-3 coding region. The WAP promoter was a 2.6-kb DNA corresponding to the 5' end of the WAP gene extending into the first exon just 5' of the first AUG as described[20,21]. The WAP-ST-3 construct was then injected into 300 embryos and implanted into 10 mice. Following a screening and breeding plan similar to that described previously for the RSV-ST-3 construct, eight positive mice were obtained with the transgene integrated into the founder germlines as described previously. When tissues from transgenic mice were examined by RT-PCR and Northern blot analysis (data not shown; see previous report), ST-3 was found to be highly expressed in three lines (designated herein as P1, P3 and P9). As described previously, ST-3 expression was limited to the mammary gland wherein expression was readily detected from mid-pregnancy through lactation (see previous report).

a) Expression of ST-3 In Vivo and Induction of an Apoptotic Program

To first determine whether ST-3 was expressed at the protein level *in vivo*, tissues from pregnant and lactating animals were examined by immunohistochemical staining with monoclonal antibodies directed against human ST-3. As shown in Figure 1, glands from the ST-3 transgenics (but not from wild-type animals) clearly expressed ST-3 which was largely confined to duct lumens (stromal staining was nonetheless detected). Because i) we noted a heightened level of pup death in several transgenic lines wherein the newborns could be rescued by foster mothers and ii) ST-3 expression *in vivo* is associated with tissue remodeling states characterized by apoptosis, tissues from wild-type and ST-3 transgenics were examined quantitatively for apoptosis by TUNEL (as described in the previous progress report) at midpregnancy, during lactation and 3 days after weaning. As shown in Figure 2, each of the transgenic lines displayed a striking increase in apoptosis albeit at different time points. In addition, as reported in other transgenic animals which have used the WAP promoter, variability can exist even within a defined transgenic strain[25]. These studies provide the first demonstration that ST-3 can directly induce the expression of an apoptotic program *in vivo*.

b) Effect of ST-3 Expression on Epithelial Cell Growth, Basement Membrane Assembly and Branching Morphogenesis In Vivo.

Given the ability of ST-3 to induce an apoptotic program *in vivo*, we next sought to determine whether the proteinase was i) directly cytotoxic, ii) initiated apoptosis during/after branching morphogenesis (akin to the effect observed *in vivo*) or iii) affected basement membrane assembly or stability (and hence, precipitated an apoptotic program). To evaluate these functions *in vitro*, we needed to identify epithelial cells capable of undergoing branching morphogenesis *in vitro*, able to deposit a basement membrane-like structure *in vitro* and one in which stable clones overexpressing ST-3 could be readily selected. To this end, we found that a subclone of the MDCK cell line was able to fulfill all three criteria. (As ST-3 expression *in vivo* has been associated with apoptotic programs in a wide variety of epithelia, we do not believe - at least at this juncture - that the effect of ST-3 is tissue-specific.) Interestingly, MDCK cell lines overexpressing active ST-3 proliferated normally in the absence or presence of the synthetic matrix metalloproteinase inhibitor, BB-94 (Fig. 3A). Furthermore, the overexpressing cells

were able to undergo a branching morphogenesis program in response to the morphogen, scatter factor (which also initiates a similar program in breast epithelial cells[26]), indistinguishable from control MDCK (Fig. 3B). Furthermore, no increases in apoptosis were detected under either resting or branching conditions (data not shown). Finally, when we examined the effect of ST-3 on basement membrane assembly, neither assembly or stability was affected (Fig. 3C and 3D). Given the fact that purified ST-3 does not degrade known matrix molecules[16], these data suggest that direct effects of the active proteinase on cell viability or matrix synthesis do not explain the *in vivo* phenotype.

c) Effect of ST-3 on Subepithelial Basement Membrane Structure In Vivo

Recent studies have demonstrated that ST-3 has recently been localized to basement membrane surrounding carcinomatous sites *in vivo*[14]. Furthermore, Shi and colleagues have reported that during amphibian metamorphosis, tissue remodeling is accompanied by striking increases in apoptosis, ST-3 expression and alterations in basement membrane structure[27,28]. These findings have led these investigators to propose that ST-3 may indirectly alter basement membrane structure via a proteolytic mechanism which then initiates apoptosis. While our *in vitro* experiments with purified ST-3 or transfected cells did not support a model wherein the proteinase directly degraded the basement membrane, these data did not rule out the possibility that ST-3 might initiate a more complex, but indirect, cascade *in vivo*. Indeed, consistent with the changes observed in amphibian tissues during morphogenesis, transgenic glands undergoing apoptosis displayed marked changes in the surrounding basement membrane (Fig. 4). While the particular basement membrane component(s) being degraded remain undefined, immunohistochemical analyses reproducibly indicate a decrease in entactin and type IV collagen staining in the ST-3 transgenics (Fig. 5). Together, these data suggest that ST-3 can indirectly mediate basement membrane dissolution *in vivo* and engages a pro-apoptotic program in normal epithelia.

ST-3 AND ANGIOGENESIS

While examining whole mounts of lactating transgenic mice, we noted a marked increase in gross vascularity (see earlier progress Report). These data suggest that ST-3 either directly stimulates angiogenesis or alternatively, induces an angiogenic response secondary to the initiation of a premature involution program during lactation. Because a role for ST-3 in angiogenesis has not been previously predicted, we sought to determine whether the proteinase is normally expressed during neovascularization. To this end, we constructed an *in vitro* model of angiogenesis wherein mouse tissue explants (1 mm^3) were embedded in 3-dimensional collagen gels and cultured in Transwells according to a modification of the technique developed by Nicosia and colleagues[29]. As shown in our previous report, neovessels are generated during a two week incubation period wherein a complex pattern of anastomosing vessels are formed. Ultrastructural studies have demonstrated that the neovessels are surrounded by pericytes, linked by tight junctions and invested with a basement membrane. Interestingly, coincident with the expression of the angiogenic phenotype, high levels of ST-3 were detected at both the mRNA and protein level. Subsequent studies revealed that ST-3 was confined to surrounding fibroblasts/myofibroblasts (and not endothelial cells) which emigrated from the tissue explant

along with the sprouting microvessels. To determine whether ST-3 plays a necessary role in the angiogenic process, endothelial cells were isolated and induced to undergo an invasive program in the presence or absence of ST-3-producing fibroblasts. Interestingly, the fibroblasts did not play a necessary role in endothelial cell invasion or tubulogenesis. Thus, while these studies were largely negative (but confirmatory of recent reports that ST-3$^{-/-}$ mice are normal and do not display defects in angiogenesis during development or wound healing[29]), They clearly demonstrate that ST-3 does not act as an angiogenic factor nor does it play a required role in blood vessel formation. A portion of these results were incorporated into a study characterizing the role of matrix metalloproteinases in angiogenesis which has been submitted for publication (see Appendix).

III. CONCLUSION

The research completed to date has allowed us to demonstrate that although human ST-3 expresses only limited proteolytic activity *in vitro*, its *in vivo* expression initiates a complex apoptosis/involution a massive program. These data provide some of the first insights into ST-3 function *in vivo* and strongly suggest that the proteinase may play an important role in regulating tumor progression by regulating basement membrane turnover. While recent studies with ST-3$^{-/-}$ mice have demonstrated that ST-3 may play a role in promoting tumor cell survival[29], our data indicate that the enzyme may actively regulate invasive processes by indirectly regulating basement membrane turnover *in vivo*. Under these circumstances, normal epithelial cells undergo apoptosis, but we propose that neoplastic cells (which resist apoptotic signals[30]) would invade and survive in the surrounding tissues.

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V. APPENDIX

1. Figure Legends and Figures

Figure 1. *Immunohistochemical localization of stromelysin-3 in transgenic mammary glands.* Mammary glands from late pregnant and lactating wild-type and transgenic mice were sectioned and stained with a polyclonal antibody to stromelysin-3. Strong expression of stromelysin-3 protein was localized to the alveolar lumens of transgenic mice at both stages (bottom row). Little or no expression was detected in wild-type mice (top row).

Figure 2. *Mammary gland apoptosis.* Mammary glands were removed from wild-type and three transgenic mouse lines (P1, P3 and P9) at different days of pregnancy (P), lactation (L) and involution (I) and stained for apoptotic cells using the TUNEL method. Apoptotic cells were counted manually and are expressed as the average number of apoptotic cells per 20 x microscopic field. Apoptosis is very low in wild-type mice throughout pregnancy and lactation and increases with gland involution. In contrast, transgenic mice often show high levels of apoptosis starting in mid to late pregnancy, with some of the highest levels found during lactation.

Figure 3. *Stromelsyin-3-transfected MDCK cells in vitro.* (A) Representative growth curve demonstrating that MDCK cells transfected with stromelysin-3 grow at the same rate as wild-type MDCKs. The synthetic MMP inhibitor BB-94 (1μm) had no effect on growth rates (asterisks indicate cell counts after seven days in the presence of BB-94). (B) Transfected cells invade collagen and undergo tubulogenesis with the addition of scatter factor (50 ng/ml), similarly to wild-type MDCK cells. In face view of ST-3-transfected MDCK cells invading into an underlying gel of type I collagen in a Transwell culture dish. (C) Transfected cells grown on collagen for three weeks form an intact basement membrane (arrows) that is indistinguishable at the EM level from the basement membrane formed by wild-type MDCKs over the same period (not shown). (D) After a 3 week incubation period, the underlying basement membrane generated by ST-3-overexpressing MDCK appeared structurally intact.

Figure 4. *TEM analysis of mammary gland after 5 days of lactation.* In panels A and B, glands from normal animals show normal alveoli with intact basement membranes. In contrast, glands from lactating transgenic animals (C and D) show marked increase in apoptotic cells (indicated by arrows) as well as changes in basement membrane structure beneath apoptotic cells (arrow in panel D).

Figure 5. *Basement membrane staining of lactating mammary glands.* Lactating glands from wild-type and transgenic mice were sectioned and stained for the major basement membrane proteins, laminin, entactin and type IV collagen.

2. Submitted Manuscript

Hiraoka, N., Allen, E., Apel, I.J., Gyetko, M.R., and Weiss, S.J. Matrix Metalloproteinases Regulate Neovascularization by Acting as Pericellular Fibrinolysins. Submitted for Publication.

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VII. Individuals Employed on Grant

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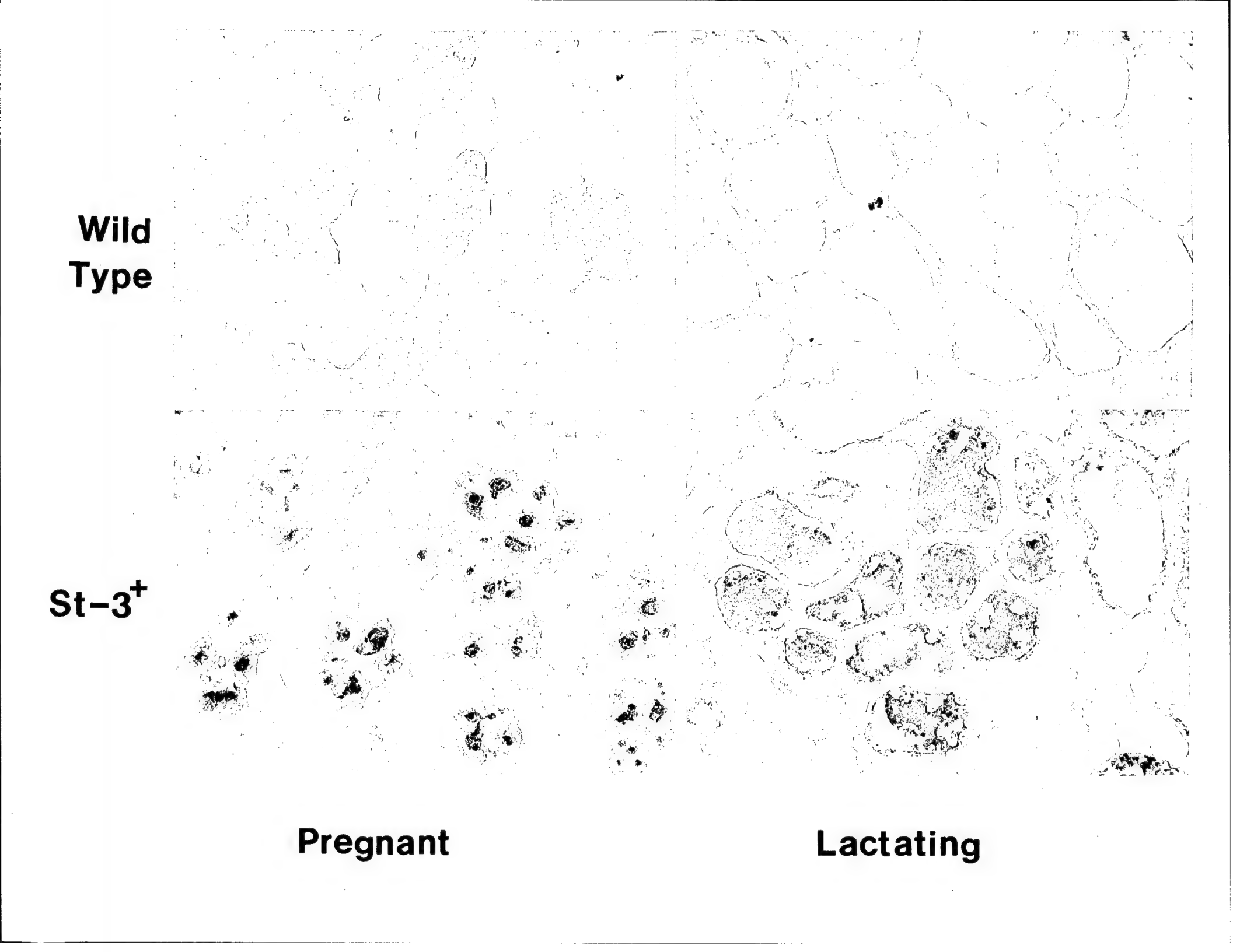
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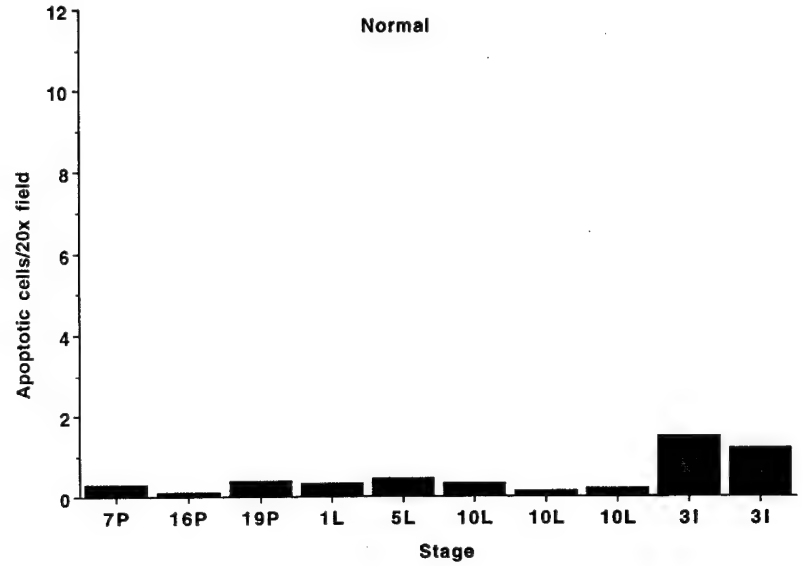
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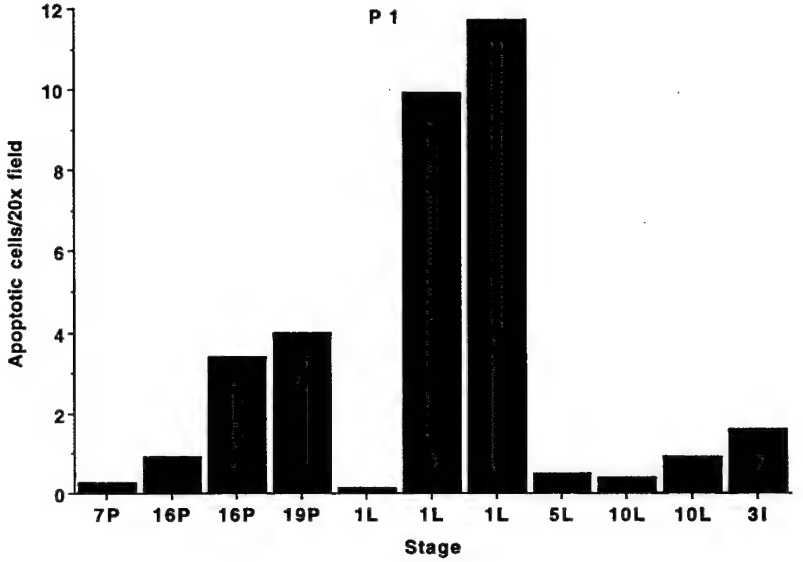
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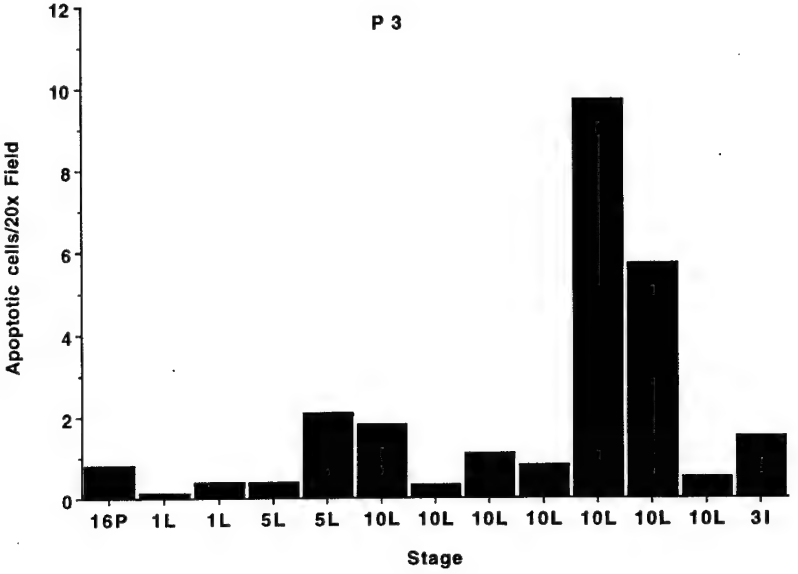
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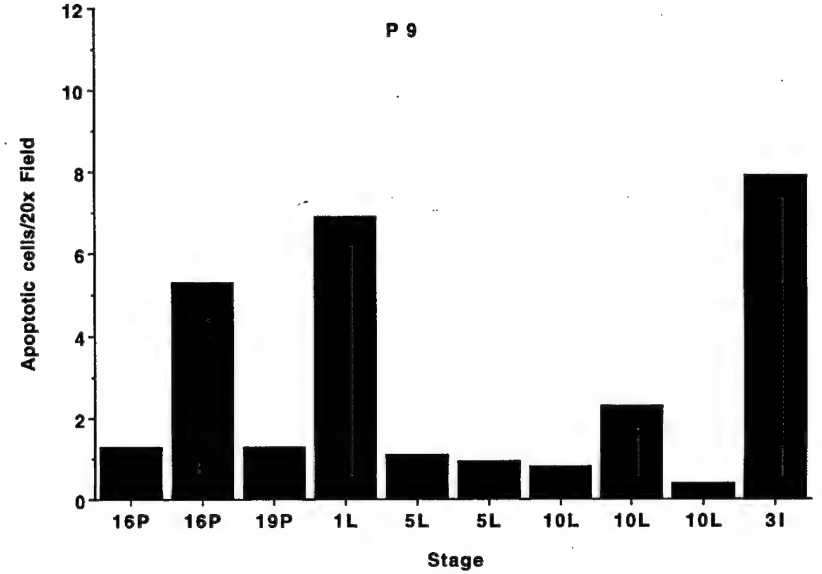
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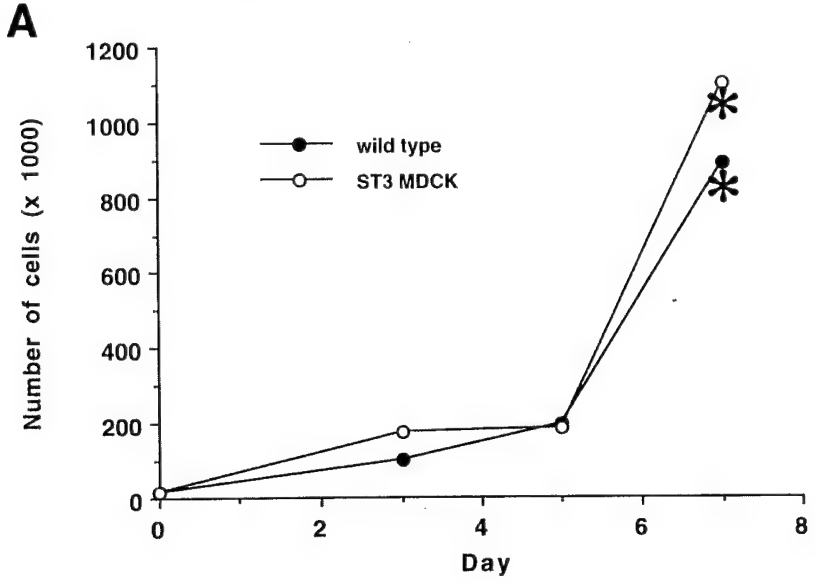
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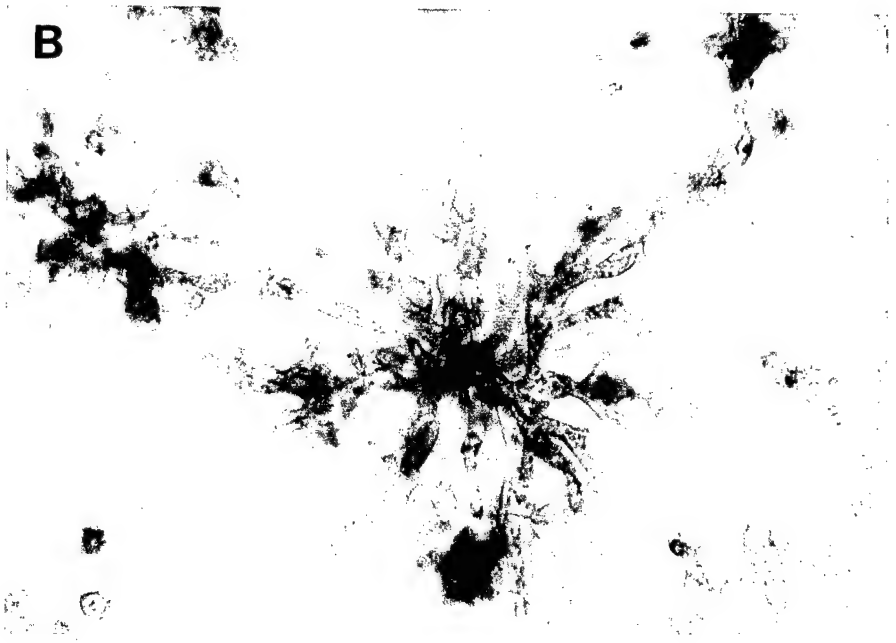
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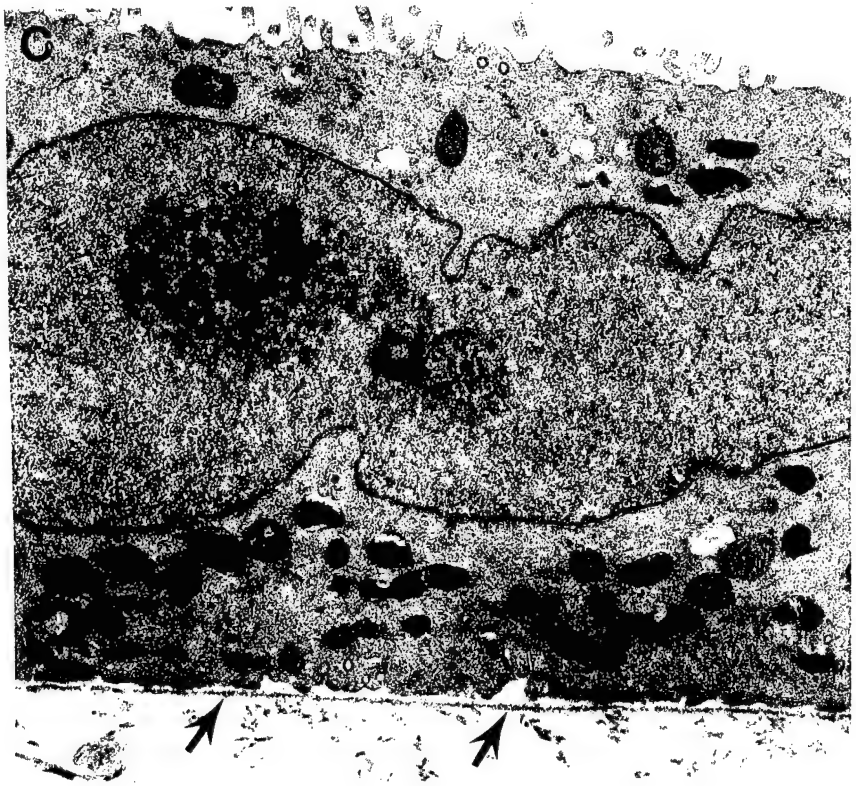
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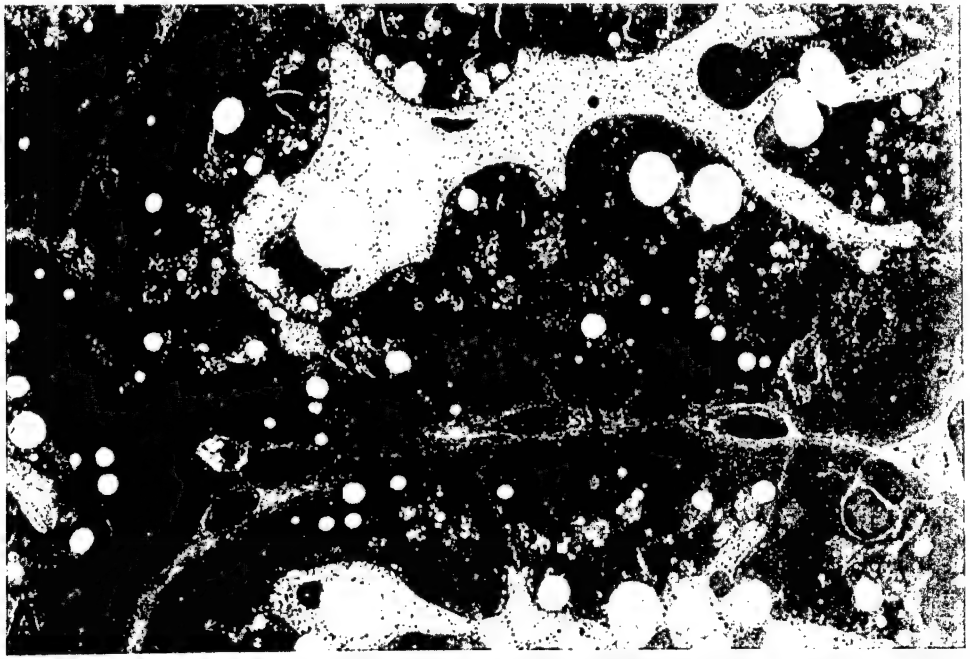

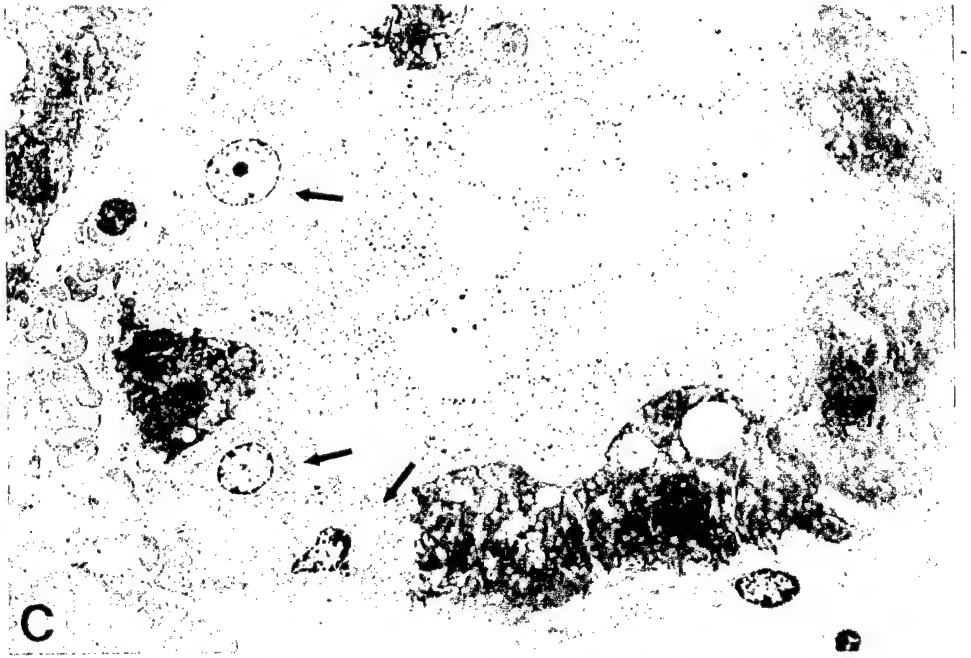
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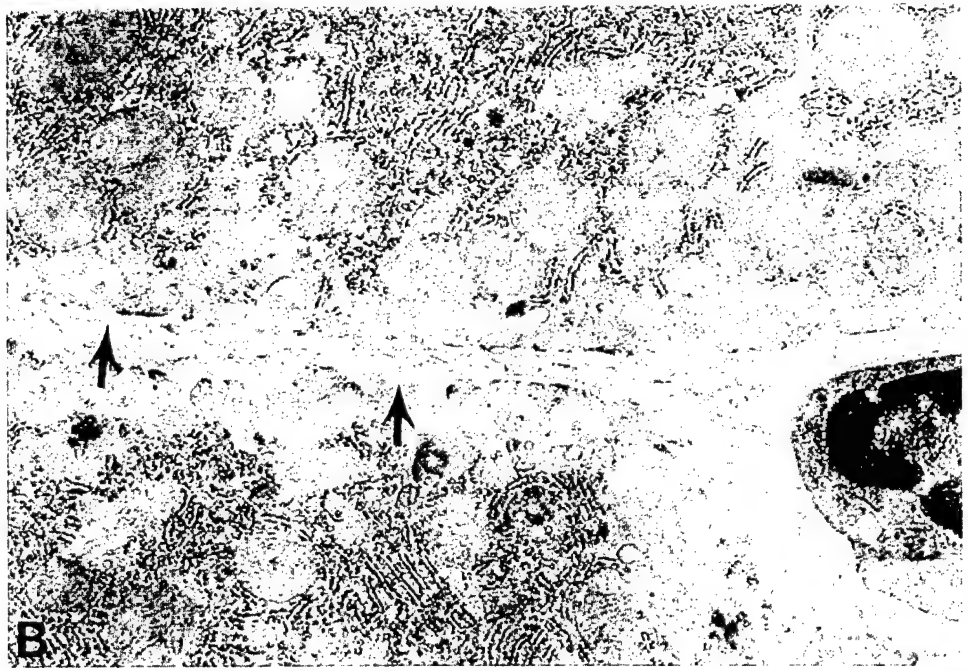
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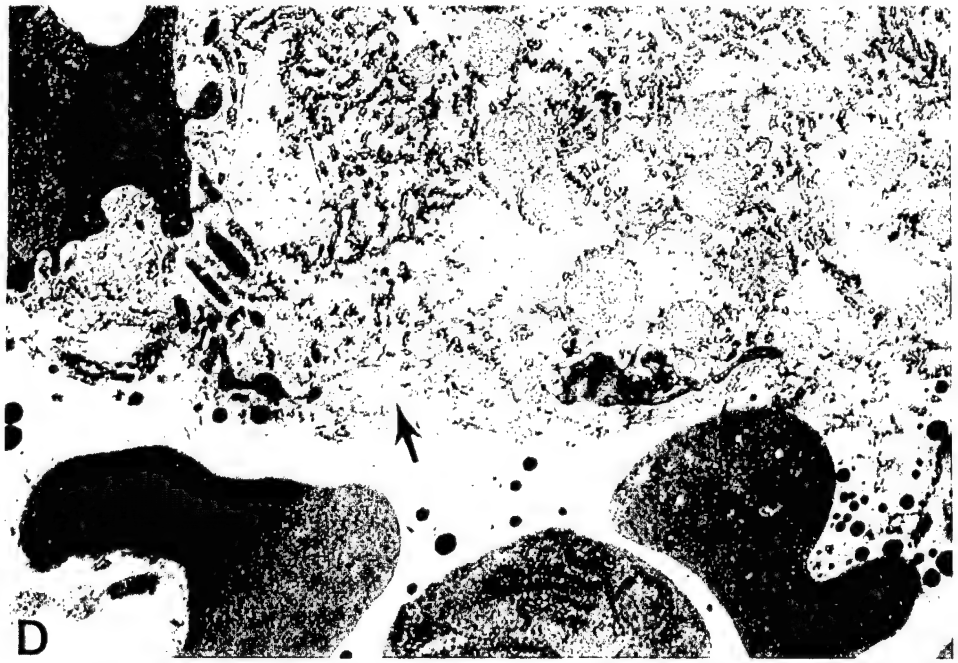
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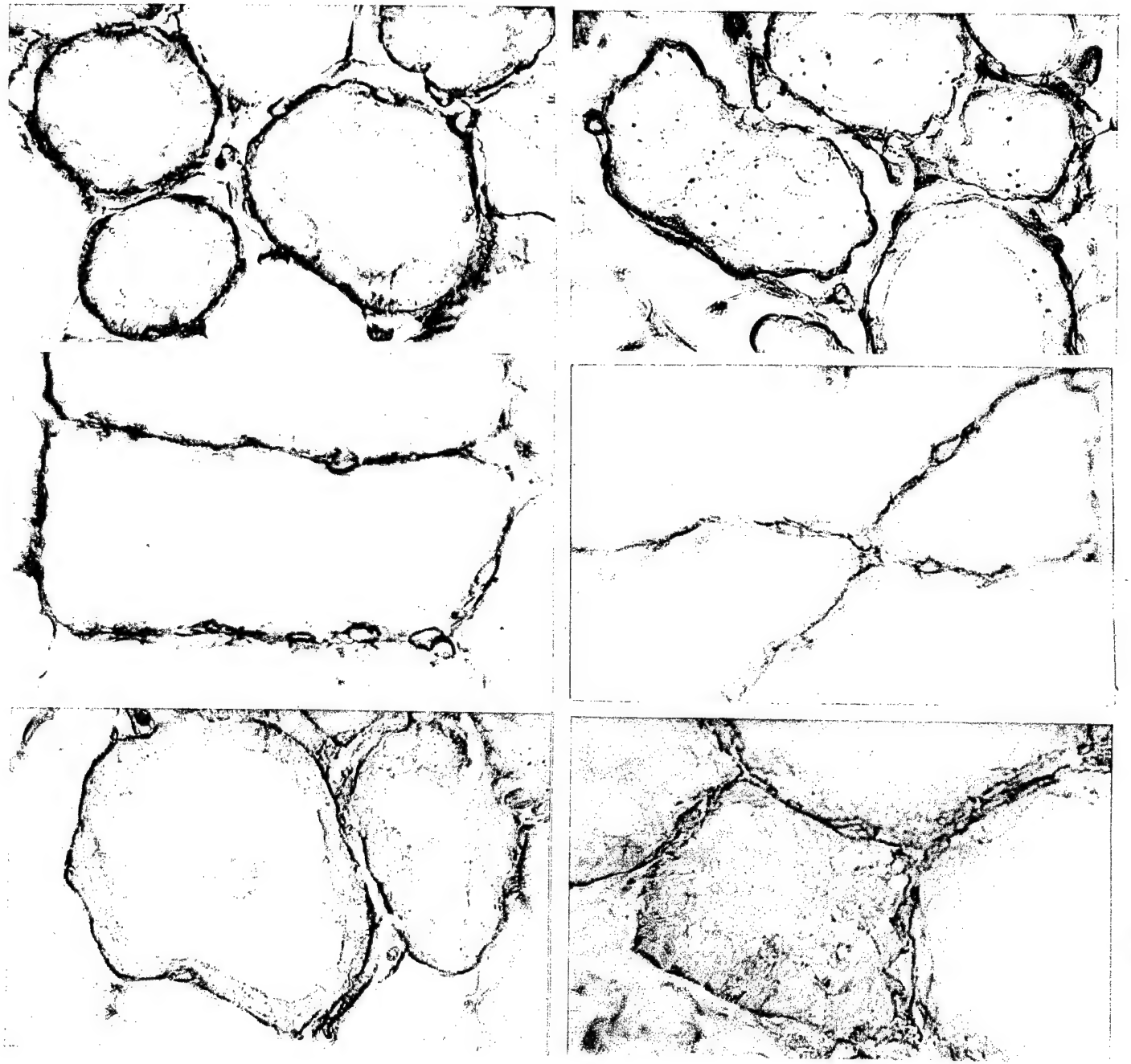

Laminin

Entactin

Collagen IV

Wild Type

Transgenic

Matrix Metalloproteinases Regulate Neovascularization by Acting as Pericellular Fibrinolysins

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Running Title: MMPs Act as Pericellular Fibrinolysins

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SUMMARY

Angiogenesis induced by wounds, tumors or inflammation requires ingressing endothelial cells to penetrate barriers of cross-linked fibrin by mobilizing undefined proteolytic mechanisms. We find that the fibrinolytic serine proteinase system comprised of plasminogen activators (PA) and plasminogen is not required for this process since tissues isolated from PA or plasminogen-deficient mice maintain a normal neovascularization response when embedded in fibrin gels. By contrast, we observe that neovessel formation, *in vitro* and *in vivo*, is completely dependent on endothelial cell-derived matrix metalloproteinases (MMP) that actively degrade fibrin matrices. MMPs play a direct role in fibrin-invasive processes as invasion-defective cells acquire the ability to penetrate fibrin gels, and exhibit tubulogenic activity, when transfected with the most potent fibrinolytic metalloproteinase identified in endothelial cells, membrane type-1 MMP (MT1-MMP). Plasma membrane display of this enzyme is critical for acquisition of the invasive phenotype, since invasion-defective cells expressing a fibrinolytically-active, but transmembrane-deleted, form of MT1-MMP remained non-invasive. These observations identify a novel plasminogen activator-independent fibrinolytic pathway that uses membrane-anchored matrix metalloproteinases as a new class of pericellular fibrinolysins essential to the neovascularization of fibrin-rich tissues.

INTRODUCTION

Pro-angiogenic signals associated with wound healing, inflammation or tumor growth induce rapid increases in vascular permeability in surrounding blood vessels (Dvorak, 1986; Eitzman et al., 1996; Risau, 1997; Senger, 1996). In turn, the blood clotting protein, fibrinogen, leaks out of the vascular bed where it is polymerized and cross-linked enzymatically into a dense fibrin gel (Dvorak, 1986; Dvorak et al., 1995; Senger, 1996). This deposited fibrin network then serves as a temporary scaffolding, or provisional matrix, for ingressing endothelial cells that subsequently engage morphogenetic programs to generate patent vessels (Folkman and D'Amore, 1996; Gailit and Clark, 1994; Risau, 1997). While the fibrin matrix serves as a structural support for neovascularization, its highly cross-linked structure also presents a major barrier to cell movement (Dvorak, 1986; Dvorak et al., 1995; Senger, 1996). To invade fibrin networks, migrating endothelial cells are believed to mobilize proteolytic enzymes whose activities are limited to pericellular compartments (Pepper et al., 1996; Werb, 1997). In this manner, sufficient fibrin is degraded to allow for the formation of endothelial-lined tunnels, while the bulk of the provisional matrix is preserved to accommodate an expanding network of new blood vessels and accessory cell populations (Gailit and Clark, 1994; Pepper et al., 1996).

To date, the ability of endothelial cells to mediate fibrinolytic activity has been largely attributed to a triad of serine proteinases which include tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and plasminogen (Bugge et al., 1995; Bugge et al., 1996; Carmeliet et al., 1994; Koolwijk et al., 1996; Ploplis et al., 1995; Sabapathy et al., 1997). While neither tPA nor uPA degrades fibrin directly, both enzymes catalyze the processing of plasminogen to the powerful fibrinolysin, plasmin. Consistent with the fibrinolytic potential of

the plasminogen activator (PA) system, numerous cell types (including keratinocytes, smooth muscle cells and inflammatory cells) display major defects in their ability to invade or degrade fibrin deposits in PA- or plasminogen-deficient states *in vivo* (Bugge et al., 1996a,b; Carmeliet et al., 1994; Carmeliet et al., 1997a,b; Carmeliet et al., 1998; Romer et al., 1996). However, in these same animals, gross alterations in either the neovascularization of fibrin-rich tissues or the re-endothelialization of damaged vessel walls have not been noted (Bugge et al., 1996a,b; Bugge et al., 1997; Carmeliet et al., 1997b; Carmeliet et al., 1998; Romer et al., 1996). Consequently, increased interest has focused on the possibility that endothelial cells do not rely on the PA-plasminogen system to breach fibrin barriers *in vivo* and that alternate, but uncharacterized, fibrinolytic proteinases exist (Bugge et al., 1996; Bugge et al., 1997).

We now report the identification of a PA- and plasminogen- independent proteolytic system that allows endothelial cells to invade and neovascularize fibrin-rich tissues both *in vitro* and *in vivo*. Unexpectedly, the endothelial cell-associated fibrinolytic activity associated with invasive activity is not mediated by serine, cysteine or aspartate proteinases, but instead, by an enzyme belonging to the matrix metalloproteinase (MMP) gene family. Further studies demonstrate that membrane-type 1 matrix metalloproteinase (MT1-MMP), a member of a new subclass of membrane-anchored MMPs (Basbaum and Werb, 1996; Birkedal-Hansen, 1995; Werb, 1997), can degrade cross-linked fibrin gels and directly mediate invasive as well as tubulogenic programs. Together, these studies identify matrix metalloproteinases as a required, and previously unsuspected, component of the PA- and plasminogen-independent fibrinolytic program engaged by endothelial cells during angiogenic processes.

RESULTS

PA-Plasminogen Deficiency Does Not Affect Fibrin Neovascularization

Defects in the neovascularization of fibrin-rich deposits cannot be assessed accurately *in vivo* as new blood vessels may reach target tissues by circumnavigating those areas containing the highest concentration of the cross-linked gel (Bugge et al., 1996b; Bugge et al., 1997; Romer et al., 1996). Consequently, an *ex vivo* model of neovessel formation was developed wherein tissue explants from normal or transgenic animals were embedded in uniform fibrin gels prepared at physiologically relevant concentrations (Figure 1A). During the first four days of culture, fibroblast-like mesenchymal cells alone migrate into the gel (data not shown). Subsequently, patent tubules begin to form on day 5 with peak neovascularization occurring by day 12 where 141±21 tubules/explant were formed (mean ±1 SD of 10 experiments; Figure 1B). At this time, an anastomosing network of patent, endothelial-lined neovessels was formed and surrounded by α-smooth muscle actin-positive, fibroblast-like cells (Figure 1C-D). As observed *in vivo* (Gailit and Clark, 1994; Nicosia and Madri, 1987), the endothelial cells lining the vessels expressed junctional complexes and deposited a basement membrane-like matrix (Figure 1E-F).

To determine whether the remodeling of the fibrin matrix that accompanied endothelial cell invasion and tubulogenesis was dependent on either of the PA systems or plasminogen, explants were cultured in the presence of i) neutralizing antibodies directed against murine tPA or uPA, ii) soluble recombinant uPA receptor (to competitively inhibit uPA binding to the uPA receptor), or iii) recombinant plasminogen activator inhibitor-2 (PAI-2; an inhibitor of two-chain uPA and tPA) (Pepper et al., 1996; Yebra et al., 1996). However, regardless of whether these inhibitors

were added individually or in combination, neovessel formation progressed in a manner indistinguishable from that observed in control explants (Figure 2A-B).

To exclude the possibility that critical quantities of uPA or tPA escaped inhibition in sequestered environments formed at the endothelial cell-fibrin interface, tissue explants from mice with combined deficiencies in uPA and tPA were embedded in fibrin gels and the angiogenic response monitored. Wild-type mouse explants generated fewer neovessels than rat explants (36±6 neovessels/explant; n=5), but no significant alterations in vessel number, length, diameter or ultrastructure were observed when the responses of wild-type or $uPA^{-/-}/tPA^{-/-}$ tissues were compared directly by either light or transmission electron microscopy (Figures 2C-F). Of note, neovessel lumens were clear of fibrin deposits despite the absence of a functional PA system (Figures 2C,F). Similar results were obtained when the fibrin concentration was increased from 3 mg/ml to 6 mg/ml.

While uPA and tPA act as the primary activators of plasminogen, alternative pathways exist that might support PA-independent plasmin formation (Bugge et al., 1996b). Thus, explants from $plasminogen^{-/-}$ mice were also examined for their ability to neovascularize fibrin gels. Consistent with the results obtained with $uPA^{-/-}/tPA^{-/-}$ tissues, the angiogenic response was unaffected by the absence of plasminogen (Figures 2G,H). Similarly, potent inhibitors of plasmin-mediated fibrinolysis (i.e., aprotinin or ε-aminocaproic acid) (Pepper et al., 1996) did not affect neovessel formation by either wild-type, $uPA^{-/-}/tPA^{-/-}$ or $plasminogen^{-/-}$ explants (data not shown). Thus, neither uPA, tPA nor plasminogen played a required role in the neovascularization of fibrin matrices by ingressing endothelial cells.

Matrix Metalloproteinases Regulate Neovessel Formation *In Vitro* and *In Vivo*

To identify PA- or plasminogen-independent proteinases that mediated fibrin-invasive or tubulogenic activity, explants were first cultured in the presence of broad-spectrum inhibitors directed against serine proteinases (soybean trypsin inhibitor and α1 proteinase inhibitor), cysteine proteinases (E-64) or aspartate proteinases (pepstatin A) (Reddy et al., 1995). However, none of these inhibitors alone or in combination affected neovessel formation (data not shown).

Endothelial cells, as well as other mesenchymal cell populations, can also synthesize a variety of secreted and membrane-anchored proteinases belonging to the MMP gene family (Basbaum and Werb, 1996; Birkedal-Hansen, 1995; Werb, 1997). Like the PA-plasminogen axis, MMPs have also been implicated in matrix remodeling events, but neither endothelial cells nor any other cell type have been shown to use this class of proteinases to penetrate fibrin barriers. To determine whether MMPs were expressed during the neovascularization of fibrin gels, total RNA was extracted from either freshly isolated or 6- to 12-day old fibrin-embedded explants and assessed by RT-PCR. While gelatinase A/MMP-2 and membrane-type matrix metalloproteinase-1 (MT1-MMP) were detected in both control and neovascularizing explants, tissues actively engaged in the angiogenic response additionally expressed gelatinase B/MMP-9, stromelysin-1, stromelysin-2, stromelysin-3, matrilysin and metalloelastase.

Given the active regulation of a complex MMP program during the angiogenic response, we sought to determine whether MMP inhibitors would alter neocapillary development in the

explant model. As shown in Figure 3, tissue inhibitor of metalloproteinases-2 (TIMP-2) or the hydroxyamate-based synthetic inhibitor, BB-94 (Basbaum and Werb, 1996; Birkedal-Hansen, 1995; Botos et al., 1996), completely blocked neovessel formation in the course of a 12-day culture period in fibrin gels (Figures 3A-D) or in plasma clots (data not shown). However, non-endothelial mesenchymal cells (i.e., predominately fibroblasts and myofibroblasts as defined by smooth muscle cell actin-staining; Nicosia and Tuszynski, 1994) retained the ability to populate the surrounding matrix in the presence of MMP inhibitors (Figures 3B,D). When either wild-type, $uPA^{-/-}/tPA^{-/-}$ or $plasminogen^{-/-}$ explants were substituted for rat tissues, neovessel formation, but not fibroblast/myofibroblast migration, was similarly blocked by BB-94 or TIMP-2.

The ability of MMP inhibitors to selectively block endothelial cell invasion suggested that MMPs regulate fibrin-invasive activity in a cell-specific manner. To confirm this possibility, explant-derived fibroblasts and myofibroblasts, or endothelial cells were isolated and re-embedded separately in fibrin gels. In the presence of MMP inhibitors, explant-derived fibroblasts and myofibroblasts underwent a neuritic-like change in cell shape, but continued to express an invasive phenotype (Figure 3F). However, under the same conditions, the addition of MMP inhibitors completely blocked the invasion of the isolated endothelial cells into the surrounding fibrin gel (Figures 3G,H). Thus, while the ability of fibroblasts/myofibroblasts to mount a PA-, plasminogen- or MMP-independent migratory response is consistent with a non-proteolytic, mechanical process (Brown et al., 1993), endothelial cell invasion was dependent on MMP activity.

Because these *ex vivo* studies supported a primarily role for MMPs rather than the PA-plasminogen axis in regulating endothelial cell invasion and tubulogenesis, a requirement for MMPs in the neovascularization of fibrin matrices was tested *in vivo*. Thus, fibrin gels or plasma clots were mixed with a cocktail of pro-angiogenic factors (see Methods) in the absence or presence of TIMP-2, sealed in perforated plastic chambers and implanted subcutaneously in wild-type mice. After a 10 day incubation period, the chambers were recovered and the angiogenic response assessed in tissue sections. Macroscopically, both the TIMP-free and TIMP-containing chambers were surrounded by large numbers of neocapillaries *in situ*. However, when the chambers were freed of surrounding connective tissues and opened, striking differences in the vascularization of the implanted fibrin gels were noted. As shown at both the light and TEM levels, while TIMP-free chambers were infiltrated with large numbers of neovessels, no blood vessels were identified in the presence of TIMP-2 in either fibrin implants (Figure 4A-D) or plasma clots (data not shown). As observed in the *in vitro* explant model, non-endothelial mesenchymal cells retained the ability to infiltrate the fibrin gels in the presence of the MMP inhibitor (Figures 4B,D). Taken together, these data support the contention that MMPs play a required role in the neovascularization of fibrin-rich tissues *in vitro* as well as *in vivo*.

Endothelial Cell MMPs Express PA- and Plasminogen-Independent Fibrinolytic Activity

If endothelial cells use MMPs as direct-acting fibrinolysins, i) the onset of invasive activity should coincide with the MMP-dependent degradation of the fibrin substratum and ii) one or more of the subset of MMPs expressed by the invading endothelial cells should display fibrinolytic activity under cell-free conditions. In the course of a 12-day incubation, growth factor-stimulated endothelial cells invaded an underlying [^{125}I]-labeled fibrin gel (Figures 5A-D)

and increased the solubilization of the substratum by three-fold in the presence of aprotinin (i.e., from 642±195 to 1596±468 cpm; mean ±1 SEM of 3 experiments). However, when endothelial cells were stimulated in the presence of either BB-94 or TIMP-2, endothelial cell-mediated fibrinolysis was reduced to resting levels (i.e., 98% ±3% and 94% ±12% inhibition of fibrinolysis; n=3) and endothelial cell invasion of the underlying fibrin gel was inhibited completely (Figures 5E,F). Invasion was similarly inhibited by BB-94 or TIMP-2 when endothelial cells were stimulated to invade fibrin gels cultured in the absence of aprotinin (Figures 5G,H). While MMP inhibitors may have affected invasion by modifying cell motility or proliferation (Corcoran and Stetler-Stevenson, 1995; Pilcher et al., 1997), neither BB-94 nor TIMP-2 inhibited endothelial cell migration across fibrin-coated surfaces (i.e., control cells stimulated with angiogenic factors migrated 1.1±0.1 mm in the absence of inhibitors and 1.4±0.1 and 1.1±0.1 mm in the presence of BB-94 or TIMP-2, respectively; n=3, mean ±1 SD) or endothelial cell proliferation in the presence of angiogenic factors (i.e., after a 3 day incubation, endothelial cell numbers increased from $1.0x10^6$ cells to 7.5 $\pm 1.4x10^6$, 7.0 $\pm 1.0x10^6$ and 9.1 $\pm 0.2x10^6$ when cultured alone, with BB-94 or TIMP-2, respectively; n=3, mean ±1 SD).

Coincident with the invasive process, isolated endothelial cells expressed a limited subset of the MMPs detected in the more complex *ex vivo* model which included collagenase-1, stromelysin-1, MT1-MMP and gelatinase A/MMP-2 (Figure 6A). To determine if these enzymes exhibit fibrinolytic activity, collagenase-1, stromelysin-1, gelatinase A/MMP-2 or a soluble, transmembrane-deletion mutant of MT1-MMP (Pei and Weiss, 1996) were incubated with [^{125}I]-labeled, cross-linked fibrin gels and solubilization quantified. Under cell-free conditions, each of the MMPs solubilized the fibrin gels via a process that could be completely blocked by BB-94

or TIMP-2 (Figure 6B). SDS-PAGE analysis of fibrin(ogen) following incubation with soluble MT1-MMP, the most potent fibrinolytic MMP identified, demonstrated that whereas the Aα and Bβ chains of fibrinogen were sensitive to proteolytic attack, α-fibrin and the high molecular weight α-polymers were preferentially degraded in the cross-linked gels (Figure 6C). Qualitatively similar results were obtained with stromelysin-1, collagenase-1 or gelatinase A (data not shown).

MT1-MMP Regulates Fibrin-Invasive Activity and Supports Tubulogenesis

Given that stromelysin-1 and MT1-MMP expressed the strongest fibrinolytic activity under cell-free conditions, the ability of each of the enzymes to regulate cellular invasion through fibrin matrices was examined. Taking advantage of the recent generation of stromelysin-$1^{-/-}$ mice (Mudgett et al., 1998), tissue explants derived from these animals were embedded in fibrin gels and neovessel formation monitored. Significantly, stromelysin-$1^{-/-}$ explants initiated an angiogenic response equivalent to stromelysin-$1^{+/+}$ control explants when assessed quantitatively or by morphologic criteria (Figure 7A).

As stromelysin-1 was not required for neovessel formation, we next sought to determine if MT1-MMP could play a direct role in pericellular fibrinolysis by endowing an invasion-null cell with invasive or tubulogenic properties via *de novo* expression of MT1-MMP. Thus, control or MT1-MMP stable transfectants were seeded atop fibrin gels and stimulated with the motogen, scatter factor, in order to elicit a motile phenotype. As shown in Figure 7B, control MDCK cells were unable to invade the fibrin gels following a six day incubation period. In marked contrast, MT1-MMP transfectants not only invaded the underlying fibrin matrix, but also underwent a

tubulogenic response reminiscent of that observed with endothelial cells (Figure 7C). Furthermore, the invasive/tubulogenic response obtained with MT1-MMP-transfected cells was blocked completely by either BB-94 (Figure 7D) or TIMP-2 (data not shown). While MT1-MMP-transfected cells process progelatinase A/MMP-2 to its active form (Butler et al., 1998; Sato et al., 1994), the MCDK clones used in these studies do not express gelatinase A/MMP-2 and invasion proceeded normally in the presence of gelatinase A-depleted serum (Figure 7E).

As MT1-MMP contains both transmembrane and cytosolic domains that do not play a direct role in controlling proteolytic activity (Ohuchi et al., 1997; Pei and Weiss, 1996), the ability of wild-type MT1-MMP to regulate fibrin-invasive activity was directly compared to mutant forms of the MMP in which either the cytosolic domain (MT1-MMP Δcyt) or transmembrane domain were deleted (MT1-MMP ΔTM). Similarly, MT1-MMP Δcyt expressed an invasive phenotype indistinguishable from the full-length enzyme (Figure 7F). However, despite the fact that MT1-MMP ΔTM-transfected cells secrete large amounts of the fully active enzyme extracellularly (Pei and Weiss, 1996), the soluble enzyme was unable to direct the invasive phenotype under conditions permissive for either of the membrane-anchored forms of the proteinase (Figure 7G). Thus, the ability of MT1-MMP to direct invasive activity was critically dependent on its localization to the cell surface.

DISCUSSION

The deposition of a fibrin-rich matrix is tightly linked to the initiation and propagation of angiogenic responses in most, if not all, post-developmental states (Dvorak, 1986; Senger, 1996; Dvorak et al., 1995). Nonetheless, the proteolytic processes mobilized during neovessel formation have remained the subject of uncertainty (Bugge et al., 1996b; Bugge et al., 1997; Pepper et al., 1996; Romer et al., 1996). In order for the fibrin matrix surrounding angiogenic sites to serve as a scaffolding for ingressing blood vessels, invading endothelial cells restrict fibrinolytic activity to the immediate pericellular milieu so that the bulk of the fibrin matrix is retained as a structural support. At the cell surface, PAs not only may regulate adhesion, migration and gene expression (Chapman, 1997), but also catalyze the formation of plasmin. In turn, plasmin efficiently degrades fibrin (Bugge et al., 1995; Bugge et al., 1996; Romer et al., 1996), extracellular matrix components (Chen and Strickland, 1997) and processes MMP zymogens to active forms (Carmeliet et al., 1997, 1998). Consequently, this proteolytic system had been posited to play an indispensable role in regulating fibrin-invasive activity. However, our *ex vivo* data, coupled with *in vivo* observations made in $PA^{-/-}$ or $plasminogen^{-/-}$ mice (Bugge et al., 1996a,b; Bugge et al., 1997; Romer et al., 1996) as well as plasminogen-deficient humans (Schuster et al., 1997), all support the existence of an alternate fibrinolytic program that operates in the pericellular space.

Following screening with a series of class-specific antiproteinases, the ability of endothelial cells to invade fibrin gels was found to be dependent solely on the expression of MMP activity *in vitro* as well as *in vivo*. Invading endothelial cells were subsequently found to express a number of secreted MMPs, but the cells also synthesized MT1-MMP, a member of a new subclass of

membrane-anchored MMPs (Birkedal-Hansen, 1995; Werb, 1997). Until recently, the primary function assigned to MT1-MMP was its ability to process the gelatinase A/MMP-2 zymogen to a series of enzymatically active proteinases (Basbaum and Werb, 1996; Butler et al., 1998; Sato et al., 1994). Because MMP-2 has been linked indirectly to angiogenic states and can bind to cell surfaces via the $\alpha_v\beta_3$ integrin (Brooks et al., 1996; Brooks et al., 1998), we initially considered its role as a potential cell-associated fibrinolysin. However, gelatinase A/MMP-2 did not exhibit efficient fibrinolytic activity under cell-free conditions nor did it play a required role in regulating fibrin-invasive activity despite the fact that MDCK cells express $\alpha_v\beta_3$ (Schoenenberger et al., 1994). Significantly, all of these results are consistent with reports that MMP-$2^{-/-}$ mice develop normally and can successfully neovascularize fibrin-rich wound sites *in vivo* (Itoh et al., 1997; Itoh et al., 1998).

While a MT1-MMP•gelatinase A/MMP2 couple was not a required participant in fibrin-invasive activity, recent studies have demonstrated that MT1-MMP can directly cleave a variety of extracellular matrix components (Ohuchi et al., 1997; Pei and Weiss, 1996). Given that pericellular proteolysis associated with invasive activity is most efficiently catalyzed by membrane-associated or membrane-anchored enzymes (Basbaum and Werb, 1996; Werb, 1997), we considered the possibility that MT1-MMP might function directly as the PA/plasminogen-independent fibrinolysin. MT1-MMP had not previously been known to express fibrinolytic activity, but portions of its catalytic domain bear strong homology to potent fibrinolysins belonging to a family of hemorrhagic venom metalloproteinases (Bini et al., 1996; Bode et al., 1993; Gomis-Ruth et al., 1993; Retzios and Markland, 1992). Consistent with this structural relationship, a soluble transmembrane-deletion mutant of MT1-MMP was shown to efficiently

degrade cross-linked fibrin. Furthermore, while neither MT1-MMP-deficient mice nor MT1-MMP-specific inhibitors have been developed, we demonstrated that cells incapable of penetrating fibrin matrices need only express MT1-MMP to acquire invasive as well as tubulogenic activity. The importance of displaying the metalloproteinase on the cell surface was underlined by the fact that cells overexpressing soluble MT1-MMP lost all invasive activity. A recent report has suggested that the cytosolic domain of MT1-MMP may affect the ability of the host cell to focus the enzyme to invasive fronts (Nakahara et al., 1997), but an absolute requirement for the 20-amino acid tail could not be established in our system. As such, anchoring the active enzyme to the cell surface was the sole determinant needed to transfer fibrin-invasive activity to a null-cell population.

Given a requirement for MMPs *in vitro* and *in vivo* during the neovascularization of fibrin gels, the question arises as to when and where plasmin itself participates in fibrin turnover. First, it should be stressed that while MT1-MMP is expressed in endothelial cells *in vitro* and *in vivo* (Nomura et al., 1995; Ueno et al., 1997; Zucker et al., 1995), the enzyme is not ubiquitously expressed by all cell types that confront fibrin barriers *in vivo*. Indeed, the markedly impaired ability of keratinocytes to efficiently re-epithelialize fibrin-rich wound fields in plasminogen$^{-/-}$ animals correlates with their inability to express MT1-MMP *in vivo* and stresses the importance of plasmin in regulating invasive activity in this cell population (Bugge et al., 1996b; Okada et al., 1997; Romer et al., 1996). Thus, a reliance on MMP- versus plasmin-dependent fibrinolytic processes may be dictated by the repertoire of proteinases available to the invading cell population. Second, while all of our *in vitro* and *in vivo* studies underlined a primary role for MMPs in endothelial cell-fibrin interactions, plasmin may play important roles

under other pathophysiologic conditions. For example, endothelial cell growth factors can drive distinct angiogenic programs that rely on different sets of expressed integrins (Brooks et al., 1994). Because our *in vitro* and *in vivo* models were all dependent on vascular endothelial growth factor (unpublished observation), other angiogenic stimuli might elicit a fibrin-invasive program more heavily dependent on the PA-plasminogen axis. Indeed, Koolwijk et al. have reported that isolated endothelial cells can invade fibrin gels *in vitro* via a uPA-dependent process (Koolwijk et al., 1996). Nonetheless, it should be stressed that defects in the neovascularization of fibrin-rich deposits surrounding wounds or tumors in PA- or plasminogen-null animals have not been reported (Bugge et al., 1996a,b; Bugge et al., 1997; Romer et al., 1996).

Finally, regardless of the importance of MMPs in regulating pericellular fibrinolysis, there are scenarios in which fibrin deposits must be rapidly and completely degraded. In the case of intravascular thrombi, the PA-plasminogen axis has been demonstrated to play the critical role in clearing fibrin-rich clots (Bugge et al., 1996a; Carmeliet et al., 1994; Ploplis et al., 1995). Likewise, following neovascularization *in vivo*, the remaining fibrin scaffolding is normally resorbed by a plasminogen-dependent process and coordinately replaced by a permanent collagen-based matrix (Gailit and Clark, 1994). If proteolysis of the provisional matrix is delayed by perturbing the PA-plasminogen axis, tissue reparative processes are interrupted and fibrotic responses are precipitated (Eitzman et al., 1996; Kitching et al., 1997; Xiao et al., 1997). A role for MMPs in bulk fibrin clearance has not been examined, but it is noteworthy that intravascular as well as extravascular fibrin deposits are eventually cleared, albeit with altered kinetics, in PA- or plasminogen-deficient states (Bugge et al., 1996a,b; Romer et al., 1996). The

ability of MMPs to preferentially degrade high molecular weight fibrin polymers that are normally resistant to plasmin-mediated proteolysis (Francis and Marder, 1987) raises the possibility that MMPs also participate in the bulk degradation of cross-linked fibrin deposits *in vivo*.

Membrane proteinases appear to play an increasingly important role in the proteolytic remodeling of the extracellular matrix (Werb, 1997). From this perspective, it is important to note that MT1-MMP is only one of four members of the membrane-anchored MMPs (Basbaum and Werb, 1996; Werb, 1997). Unlike some MMPs which may rely on plasmin for processing to active forms (Carmeliet et al., 1997, 1998), each of the MT-MMPs are encrypted with an Arg-X-Lys-Arg motif upstream of their respective catalytic domains (Basbaum and Werb, 1996; Maquoi et al., 1998; Pei and Weiss, 1995, 1996). This basic tetrad can act as a recognition sequence for intracellular proprotein convertases which then activate the cognate MMP zymogen by removing its regulatory prodomain (Maquoi et al., 1998; Pei and Weiss, 1995, 1996). Thus, the proprotein convertase-dependent activation of metalloproteinase zymogens may have evolved as a PA/plasminogen-independent, but complementary, proteolytic processing system. Because the substrate specificity of membrane-anchored metalloproteinases extends beyond fibrin to include a range of extracellular matrix components (Ohuchi et al., 1997; Pei and Weiss, 1996), these enzymes are likely to act as critical mediators of invasive activity by normal as well as neoplastic cells through multiple structural barriers *in vivo*. As such, therapeutic interventions aimed at MT1-MMP and related membrane-anchored proteinases (Blobel, 1997; Millichip et al., 1998; Werb, 1997) may prove useful not only in regulating angiogenesis, but also the pathologic remodeling of the extracellular matrix in tissue-destructive disease states as well.

EXPERIMENTAL PROCEDURES

Tissue Explant Model

Abdominal muscle fragments (1.5 mm) were isolated from Fischer rats, uPA$^{-/-}$/ tPA$^{-/-}$, plasminogen$^{-/-}$, stromelysin-1$^{-/-}$ or background-matched wild-type mice (Carmeliet et al., 1994; Ploplis et al., 1995; Mudgett el al., 1998) and suspended in a solution (0.9 ml) of 3 mg/ml of PA- and plasminogen-free human fibrinogen (Calbiochem). Co-polymerized gels were formed following the addition of 15 milliunits bovine thrombin (Sigma) (Brown et al., 1993) and the explants cultured for 12 days at 37° C in MCDB-131 (Clonetics) supplemented with either 20% fetal bovine serum (FBS) or 10% mouse serum collected from control or null animals. Where indicated, the fibrin gels and media were supplemented with neutralizing polyclonal antisera directed against murine uPA (5 μg/ml) or tPA (30 μg/ml), soluble rat uPA receptor (0.5 μg/ml; all from American Diagnostica), recombinant plasminogen activator inhibitor-2 (20 μg/ml; a gift from M. Baker, Univ. of Wollongong, Australia), ε-aminocaproic acid (500 μg/ml; Sigma), aprotinin (200 μg/ml; Sigma), E-64 (100 μM; Sigma), pepstatin A (50 μM; Sigma), recombinant TIMP-2 (20 μg/ml; Amgen) or BB-94 (5 μM; British Biotechnology).

Fibrin Invasion Assays

Rat aorta endothelial cells or rat explant-derived fibroblasts and myofibroblasts were isolated as described (Nicosia and Tuszynski, 1994) and cultured in MCDB-131 supplemented with 20% FBS atop or within fibrin gels in Transwell culture dishes (Costar). In studies designed to analyze the invasive properties of fibrin-embedded cells, rat aorta endothelial cells or rat explant-derived fibroblasts and myofibroblasts were cultured atop fibrin gels, 3x4 mm fragments excised and then embedded in fibrin gels as described above for the tissue explants. Endothelial

cell motility was monitored by culturing cells atop fibrin gels whose surface was partially covered with glass cloning chips. After monolayers were formed, the chips were removed leaving denuded areas wherein the distance migrated by the advancing front of cells was measured during a 24 hour incubation.

Fibrin invasion *in vivo* was assessed by placing fibrin gels (3 mg/ml) or plasma clots containing a mixture of 100 ng/ml vascular endothelial growth factor (Genentech), 10 ng/ml TGFα (Biosource), 50 ng/ml hepatocyte growth factor (Genentech), 75 μg/ml endothelial cell growth supplement (Collaborative Research) and 100 μg/ml heparin in the absence or presence of TIMP-2 (20 μg/ml) in polycarbonate chambers (Dvorak et al., 1987). The chambers were then sealed, one end of the chamber perforated to allow cell ingress and implanted subcutaneously in wild-type mice for 10 days.

MMP-Dependent Fibrinolysis

Human umbilical vein endothelial cells were isolated as described (Huber et al., 1991) and cultured in M-199 supplemented with 20% human serum atop fibrin gels prepared with [^{125}I]-labeled fibrinogen ($3x10^5$ cpm total; Amersham) in the absence or presence of 200 μg/ml aprotinin, 5 μM BB-94 or 20 μg/ml TIMP-2. Monolayers were stimulated to invade with a mixture of angiogenic factors (as described above) which were added to the lower compartment of the Transwell system. Solubilized fibrin contained in the media and in a 5 M urea extract of the fibrin gel were pooled, recovered following centrifugation (10,000 xg for 10 min.) and quantitated by γ-scintillation counting. Human endothelial cells were used in place of murine

cells for these studies given the availability of purified and recombinant human enzymes (see below).

For cell-free studies, fibrinogen or fibrin gels (20 μg each) were incubated with 10 μg/ml aprotinin and 200 ng of organomercurial-activated human fibroblast collagenase, human stromelysin-1, recombinant human gelatinase A or a catalytically active, transmembrane-deletion mutant of human MT1-MMP (Pei and Weiss, 1996). Following a 48 h incubation in 50 mM Tris-HCl, 5 mM $CaCl_2$, 200 mM NaCl and 0.2% sodium azide in the absence or presence of BB-94 or TIMP-2, proteolysis was assessed by SDS-PAGE (7.5%). To monitor fibrinolytic activity, radiolabeled fibrinogen was incorporated into the fibrin gel ($1x10^5$ cpm) and solubilized fibrin monitored in the media or urea extract as described above.

Northern Blot and RT-PCR analysis

Total RNA was isolated using TRIzol™ reagent (Life Technologies), electrophoresed in formaldehyde-agarose gels (5 μg/lane), transferred to nylon filters and hybridized with [α-^{32}P]dCTP-labeled cDNA probes for human collagenase-1, stromelysin-1, gelatinase A/MMP-2, MT1-MMP or 36B4. Reverse transcription was performed using an oligo dT primer and AMV reverse transcriptase. cDNAs of matrix metalloproteinases were amplified by PCR using specific oligonucleotide primers for gelatinase A (Genbank accession no. X71466), MT1-MMP (X83537), metalloelastase (M82831), gelatinase B (U36476), stromelysin-1 (X02601), stromelysin-2 (X64020) and matrilysin (Z11887). The identity of the isolated cDNAs were confirmed by sequence analysis as described (Pei and Weiss, 1995). Stromelysin-3 expression

was determined by Northern blot analysis using 5 μg total RNA and a human cDNA probe (Pei et al., 1994).

MDCK Cell Lines

MDCK cells (ATCC) were stably transfected with a control vector or full-length (amino acid residues 1-582), cytosol-deleted (residues 1-563) or transmembrane-deleted form (residues 1-508 or 1-538) of MT1-MMP as described previously (Pei and Weiss,1996). MT1-MMP expression was confirmed by Western blot analysis (Pei and Weiss, 1996). MDCK transfectants were cultured atop fibrin gels in the presence of 20% fetal calf serum in Transwell dishes as described above and motility stimulated with 50 ng/ml hepatocyte growth factor. Fetal calf serum was depleted of gelatinase A/MMP-2 by gelatin-sepharose affinity chromatography and confirmed by gelatin zymography (Pei et al., 1994).

Histology: Tissues were fixed with 2% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4). After post-fixation with 1% osmium tetroxide and dehydration in ethyl alcohol, the samples were embedded in epoxy resins for light (stained with 1% toluidine blue) or electron microscopy (stained with uranyl acetate and lead citrate). For immunohistochemistry, frozen sections were stained with monoclonal anti-rat RECA-1, an endothelial cell-specific antigen (Serotec Ltd.) or monoclonal anti-α-smooth muscle cell actin (Sigma) as described (Nicosia and Tuszynski, 1994). Staining was performed with a peroxidase detection kit (Vector Laboratories) using 3-amino-9-ethyl carbazole as substrate.

Animals: Animal care at the University of Michigan Department of Laboratory Animal Medicine was in accordance with the American Association for Accreditation of Laboratory Animal Care.

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FIGURE LEGENDS

Figure 1. **Neovessel Formation by Fibrin-Embedded Murine Explants**

(A,B) Rat tissue explants were isolated from abdominal wall muscle, embedded in a transparent fibrin gel and examined by phase contrast microscopy after 1 day (A) or 12 days (B) in culture at which time an anastomosing network of neovessels had formed (x120).

(C,D) Transverse light sections of 12 day old cultures stained with endothelial cell-specific, anti-RECA-1 monoclonal antibody (C) or anti-α–smooth muscle cell actin monoclonal antibody (D). Arrows indicate RECA-1-positive vessels (C) and α-smooth muscle cell actin-positive fibroblasts/myofibroblasts (D) (x300). Weak staining of endothelial cells for α–smooth muscle cell actin was observed occasionally. Transmission electron micrographs of 12 day old neovessels depict the formation of tight junctions (E, x32,000) and a basement membrane-like subendothelial matrix (F, x45,000). Arrowheads indicate tight junctions (E) or basement membrane (F).

Figure 2. **The PA-plasminogen Axis in Neovessel Formation**

(A,B) Rat muscle explants were cultured in fibrin gels alone (A), or with a mixture of anti-tPA (20 μg/ml), anti-uPA (5 μg/ml), soluble recombinant uPA receptor (0.5 μg/ml), recombinant PAI-2 (20 μg/ml) and aprotinin (200 μg/ml) for 12 days (B). In the presence of these inhibitors, the fibrinolytic activity of isolated rat endothelial cells was inhibited by 99%. No differences were detected in neovessel formation as assessed in transverse light sections (x300).

(C-F) Neovessel formation was compared in fibrin-embedded explants isolated from wild-type or $uPA^{-/-}/tPA^{-/-}$ and cultured for 12 days in their respective autologous sera (10% final). In transverse light sections (x400) or transmission electron micrographs (x25,000) of wild-type

(C,E) or $uPA^{-/-}/tPA^{-/-}$ (D,F) explants, no differences in neovessel formation or structure were observed.

(G,H) Neovessel formation by $plasminogen^{-/-}$ explants cultured in 10% autologous sera proceeded at a rate indistinguishable from controls (G). Results are expressed in the mean number of neovessels formed ±1 SEM (n=4). Neovessel structure in $plasminogen^{-/-}$ explants (H) was also comparable to controls as assessed by transmission electron microscopy (x25,000).

Figure 3. **Effect of MMP Inhibitors on Neovessel Formation *In Vitro***

(A-D) Rat tissue explants were cultured in fibrin gels for 12 days in the absence (A,C) or presence of 5 μM BB-94 (B,D). Phase contrast microscopy (x120) demonstrated that neovessel formation was completely blocked by the MMP inhibitor (A versus B). Transverse light sections (x300) confirmed the presence of patent tubules in control, but not BB-94-treated cultures (C versus D). In the presence of BB-94, infiltrating populations of RECA-1-negative/α–smooth muscle actin-positive fibroblasts/myofibroblasts were detected (data not shown).

(E-H) Isolated, rat explant-derived fibroblasts/myofibroblasts (E,F) or rat endothelial cells (G,H) were embedded in fibrin gel in the absence (E,G) or presence of 5 μM BB-94 (F,H) and examined by phase contrast microscopy (x100). Migration from fibrin gel implants (marked by asterisks) into the surrounding fibrin matrix is shown after a 7 day incubation period. While the MMP inhibitor altered the shape of the migrating fibroblasts/myofibroblasts, motility was preserved (E,F). In contrast, endothelial cell outgrowth from implants (marked by asterisks) was inhibited completely by BB-94 (G,H).

Figure 4. **Effect of MMP Inhibition on Neovascularization *In Vivo***

(A-D) Fibrin gels containing a cocktail of angiogenic factors (see Experimental Procedures) with or without TIMP-2 were sealed in perforated plastic chambers and implanted subcutaneously in wild-type mice for 10 days. Chambers were removed and processed for light (x320) or transmission electron microscopy (x7,350). While neovessels were observed in the control implants (A and C), no blood vessels were detected in TIMP-2-containing fibrin gels (B and D). In the presence of TIMP-2, non-endothelial mesenchymal cells retained invasive activity (B,D). Results are representative of 3 independent experiments. Arrowheads indicate the position of neovessels in panel A.

Figure 5. **Effect of MMP Inhibitors on the Fibrin-Invasive Activity of Isolated Endothelial Cells**

(A,B) Human endothelial cells were cultured atop fibrin gels for 12 days with aprotinin in the absence (A) or presence of a mixture of vascular endothelial growth factor, TGFα, hepatocyte growth factor, endothelial cell growth factor and heparin (B) and examined by phase contrast microscopy (x100). Sprouts of invading endothelial cells are formed below the plane of focus of the monolayer in stimulated cultures only (B).

(C-H) Transverse light sections (x200) of unstimulated monolayers (C) or cultures stimulated with growth factors in either the absence or presence of BB-94 (D and E, respectively) or TIMP-2 (F). Endothelial cell cultures stimulated with growth factors in the absence of aprotinin (G) similarly displayed invasive activity which was blocked completely by TIMP-2 (H). Double-headed arrow indicates the position of the underlying gel in panel C while arrowheads highlight invading endothelial cells in panels D and G.

Figure 6. **MMP Expression in Endothelial Cells and Fibrinolytic Activity**

(A) Northern blot analysis of MMP expression in human endothelial cells after a 3 day incubation with a mixture of vascular endothelial growth factor, TGFα, hepatocyte growth factor, endothelial cell growth supplement and heparin.

(B) Fibrinolytic activity of collagenase-1, stromelysin-1, gelatinase A or a soluble MT1-MMP transmembrane deletion mutant in the absence or presence of BB-94 (values obtained with the inhibitor are the bars shown to the right in each pair). The clear and stippled portion of each bar represents the number of counts recovered in the media or urea extract, respectively. Results are expressed as the mean ± 1 SEM cpm released from [^{125}I]-fibrin-labeled gels (n=3).

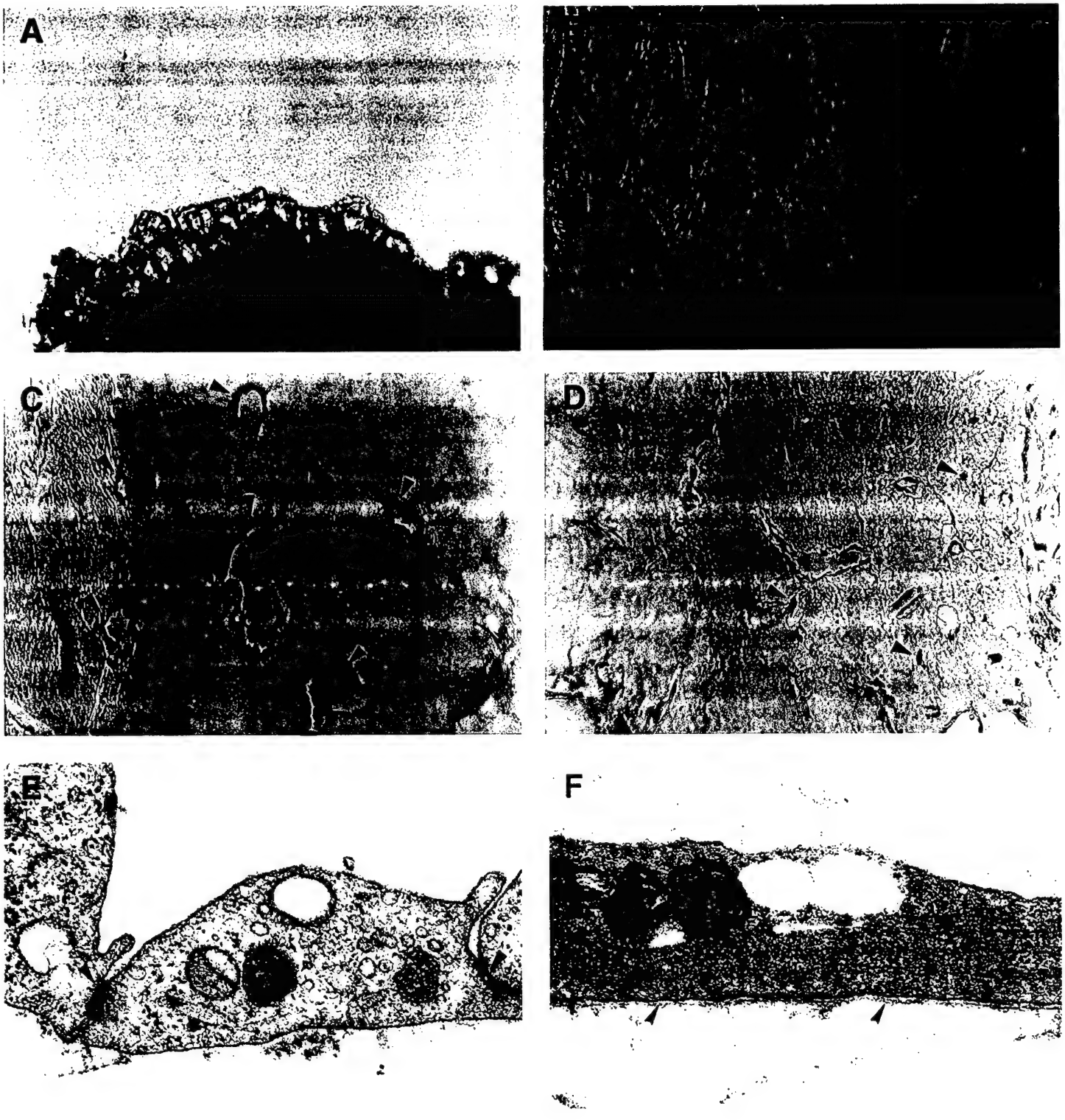
(C) SDS-PAGE analysis of fibrinogen or fibrin (20 μg each) after an 18 h incubation alone or with soluble MT1-MMP (200 ng) in the absence or presence of TIMP-2 (1 μg). Aα, Bβ and γ chains of fibrinogen and the α polymer, γγ, α and β chains of fibrin are indicated to the left of each gel.

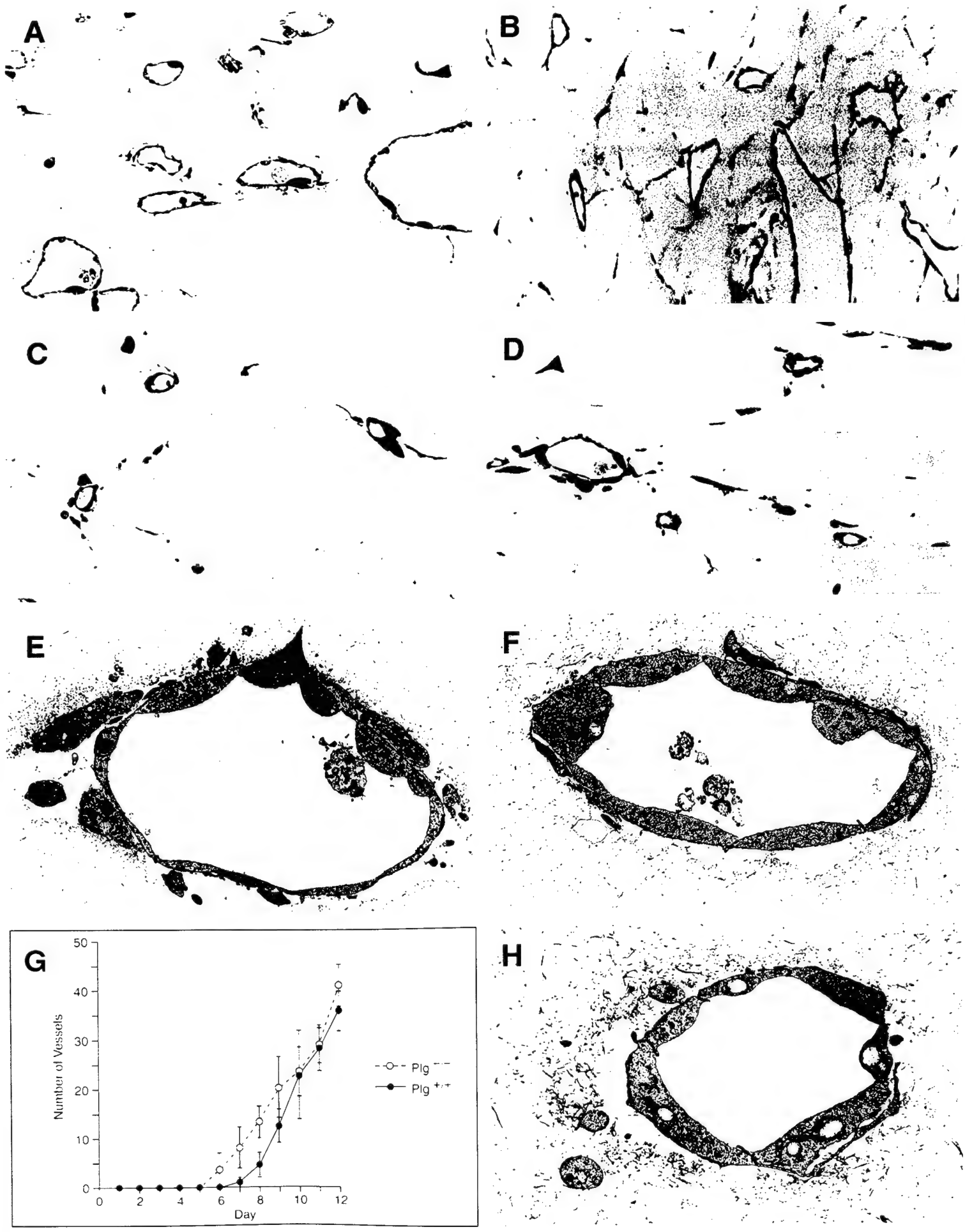
Figure 7. **Regulation of Neovessel Formation and Fibrin-Invasive Activity by Stromelysin-1 and MT1-MMP**

(A) Neovessel formation by stromelysin-1$^{-/-}$ or stromelysin-1$^{+/+}$ explants as a function of time. Results are expressed as the mean ± 1 SEM of 10 experiments with no discernible morphologic differences observed between control or null animals as shown in transverse light sections (x150).

(B-G) MDCK cells transfected with control vector did not invade fibrin gels during a 4 day incubation (B), but cells overexpressing MT1-MMP invaded the underlying fibrin and generated

tubules (C) via a BB-94-sensitive process (D). Depleting the sera of gelatinase A/MMP-2 did not alter the invasive phenotype (E). MDCK cells overexpressing MT1-MMP Δcyt, but not MT1-MMP ΔTM, retained fibrin-invasive activity (F and G, respectively). The double-headed arrow in panel B marks the position of the underlying fibrin gel while the arrows in C, E and F indicate cells invading the fibrin gel.


A
C
D
E
F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40
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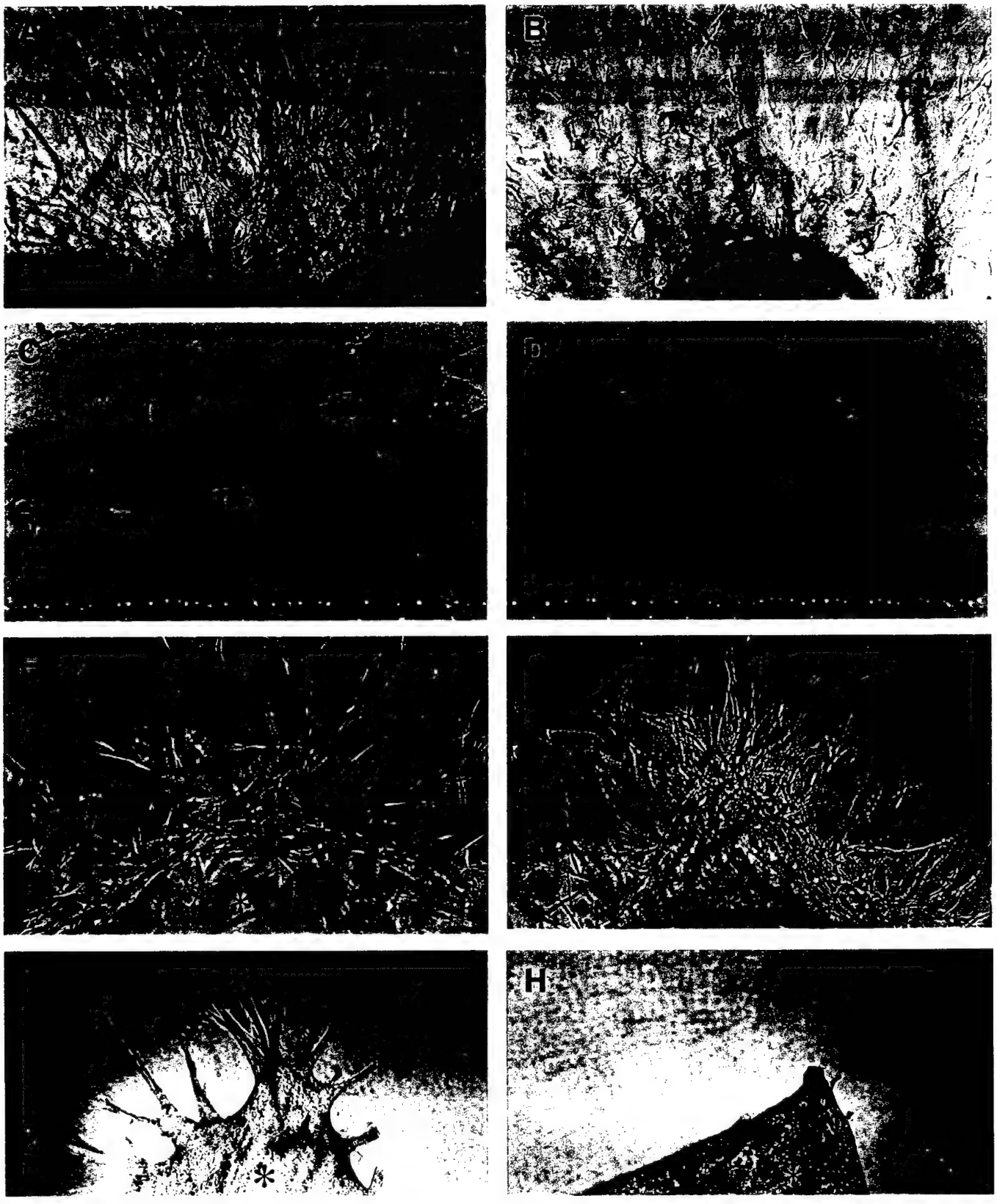
Plg -/-
Plg +/+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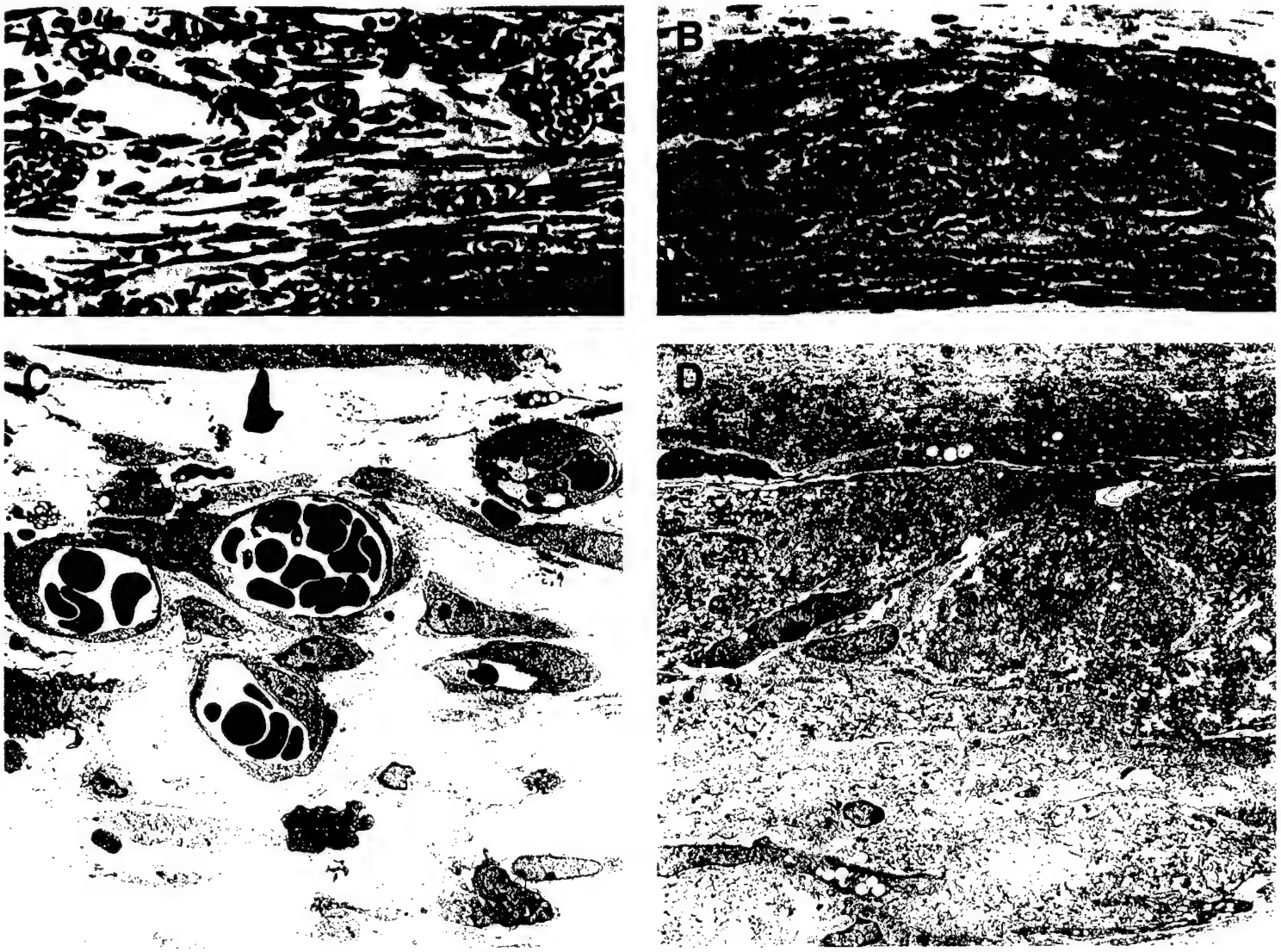
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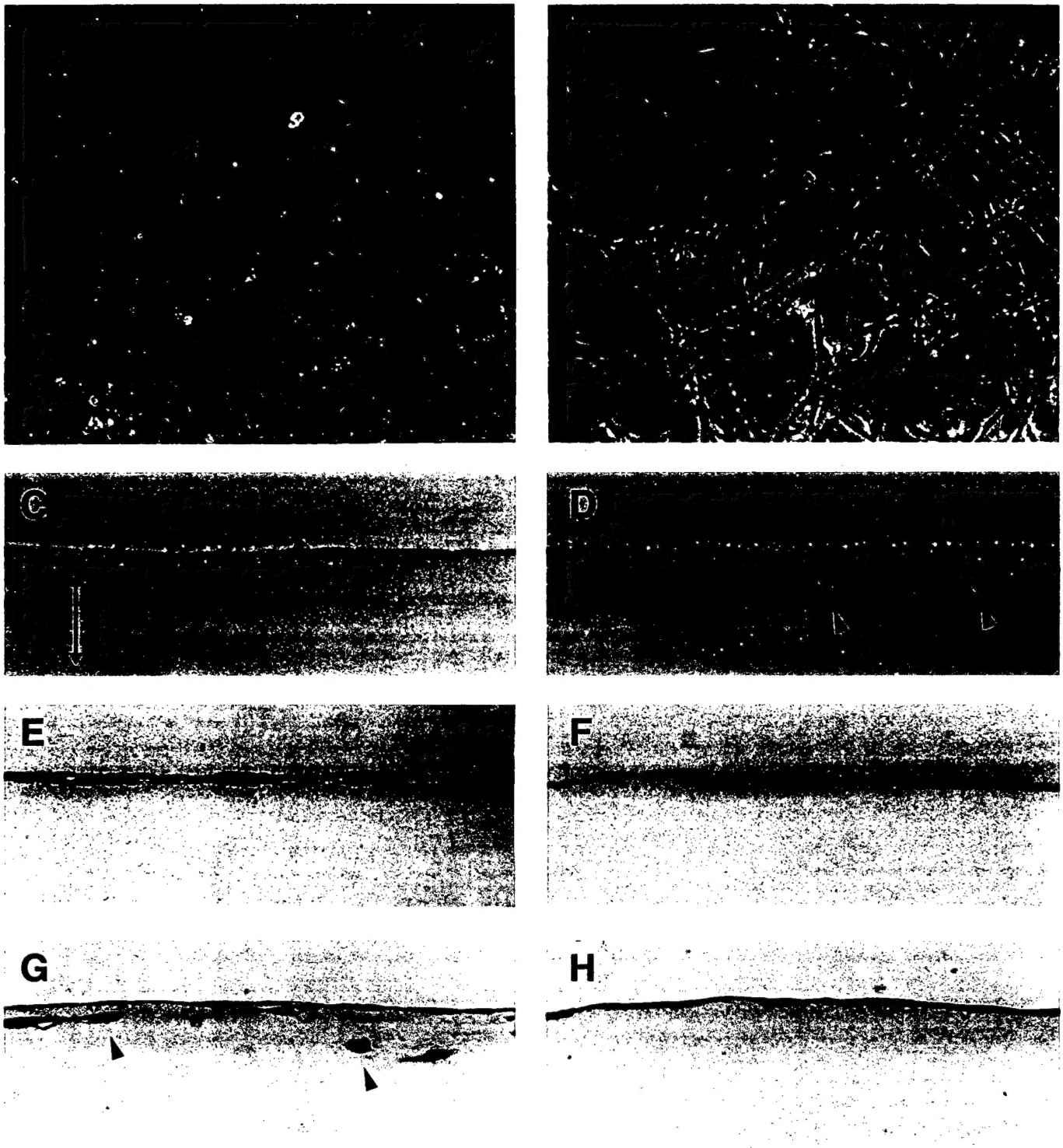
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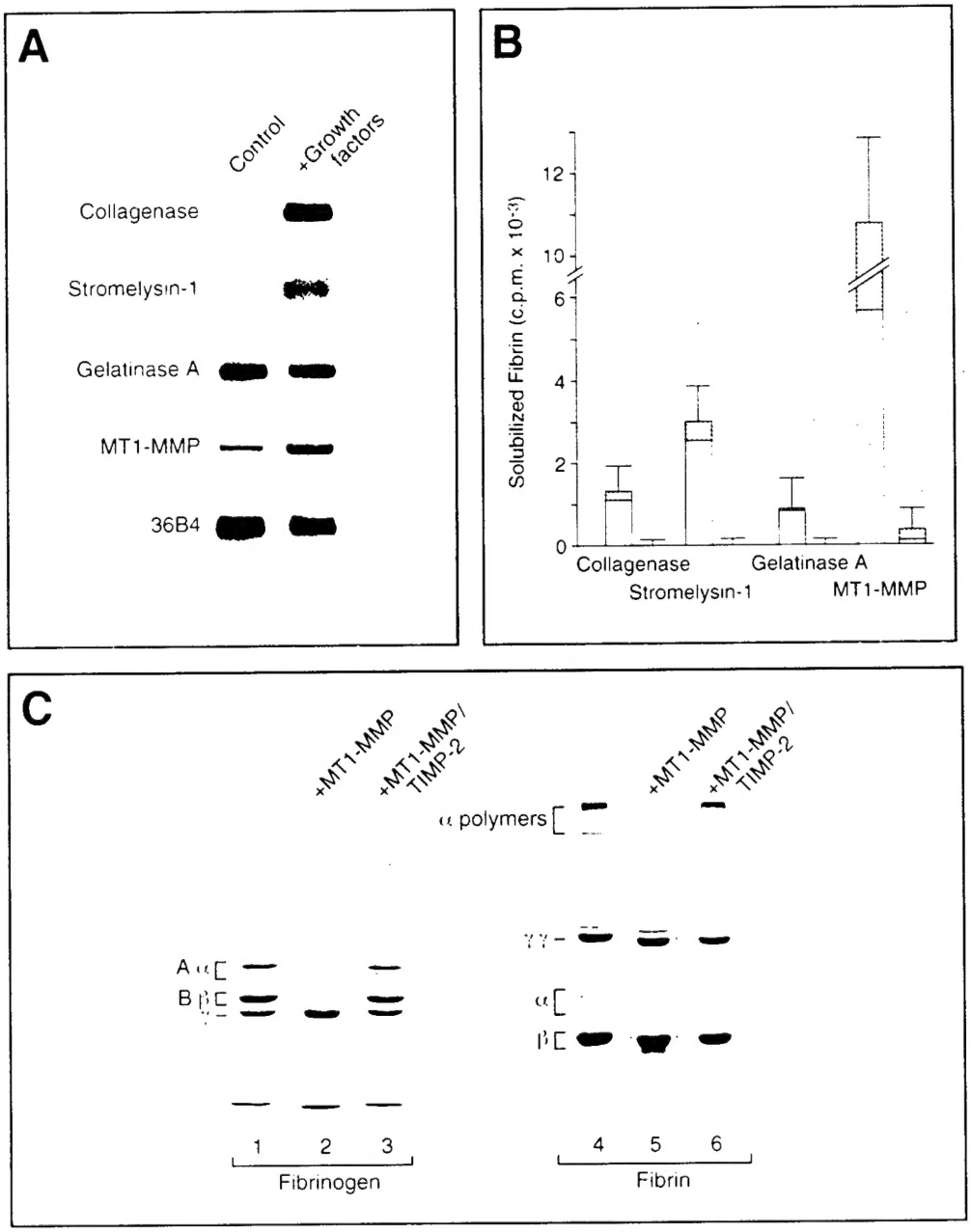
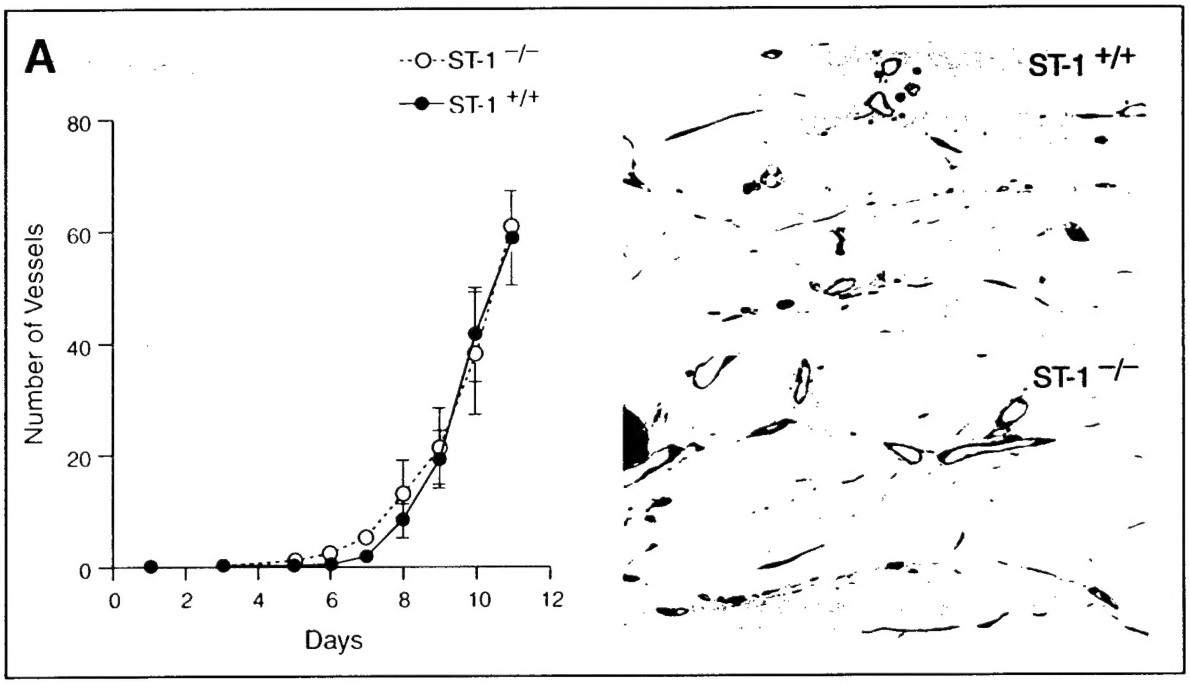

A
Control
+Growth factors
Collagenase
Stromelysin-1
Gelatinase A
MT1-MMP
36B4
B
Solubilized Fibrin (c.p.m. x 10⁻³)
12
10
6
4
2
0
Collagenase
Stromelysin-1
Gelatinase A
MT1-MMP
C
+MT1-MMP
+MT1-MMP/ TIMP-2
+MT1-MMP
+MT1-MMP/ TIMP-2
α polymers
Aα
Bβ
γγ
α
β
1 2 3
Fibrinogen
4 5 6
Fibrin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ST-1 +/+
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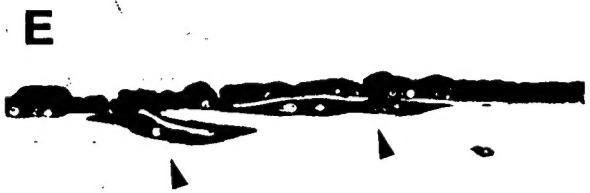
0
2
4
6
8
10
12
Days
ST-1 +/+
ST-1 −/−


B


C


D


E


F


G